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The DNA Methylation Machinery as a Target for Anticancer Therapy

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ABSTRACT. DNA methylation is now recognized as an important mechanism regulating different functions of the genome; gene expression, replication, and cancer. Different factors control the formation and maintenance of DNA methylation patterns. The level of activity of DNA methyltransferase (MeTase) is one factor. Recent data suggest that some oncogenic pathways can induce DNA MeTase expression, that DNA MeTase activity is elevated in cancer, and that inhibition of DNA MeTase can reverse the transformed state. What are the pharmacological consequences of our current understanding of DNA methylation patterns formation? This review will discuss the possibility that DNA MeTase inhibitors can serve as important pharmacological and therapeutic tools in cancer and other genetic diseases. PHARMACOL. THER. 70(1): 1-37, 1996.

KEY WORDS. DNA methylation, demethylation, *de novo* methylation, epigenetic modification, anticancer agents, 5-deoxyazacytidine.

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ABBREVIATIONS. AP, activator protein; APC, adenomatosis polyposis coli; aprt, adenosine phosphoribosyl transferase; 5-azaC, 5-azacytidine; 5-azadC, 5-deoxyazacytidine; hprt, hypoxanthine phosphoribosyl transferase; IgfII, insulin-like growth factor II; IgfIIr, insulin-like growth factor II receptor; LTR, long terminal repeat; MBDP, methylated-DNA binding protein; MeCP, methylated CpG binding protein; MeTase, methyltransferase; MoMuLV, Moloney murine leukemia virus; MyoD, myogenic differentiation; Rb, retinoblastoma; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; WT, Wilms tumor.

1. INTRODUCTION

As modern biology unravels the mysteries of how our cells and tissues interpret our genetic information, the question arises as to whether or not this understanding would help us identify important targets for pharmacological intervention that could have significant therapeutic value. Whereas pharmacology traditionally has been interested in targets controlling the physiological state of our bodies, it is clear that many disease states, such as cancer and a wide range of genetic diseases, involve alterations of the genetic program itself and should be addressed at that level. Moreover, after one understands the genetic program and the ways by which it is maintained, changed, and regulated, one might be able to replace traditional drugs that act on the periphery of tissues and cells by binding to receptors and enzymes with compounds that could alter the genetic program controlling the expression of these biomolecules. In this review, I discuss one level of regulation of genomic functions that is a good candidate to serve as an important target for anticancer and other therapies that involve modification of genetic programs. The candidate mechanism is DNA methylation and the enzymes that control DNA methylation. I will discuss the fundamental aspects of DNA methylation and how one can utilize this understanding toward the identification of pharmacological targets for anticancer therapy.

2. WHAT IS DNA METHYLATION?

Many, but not all, organisms, from bacteria to humans, contain bases modified by methylation in their genome, in addition to the basic four bases that encode the genetic information (Hotchkiss, 1948; reviewed by Razin and Szyf, 1984). In bacteria and some lower eukaryotes, both 6-methyladenine and 5-methylcytosine could be found (Razin and Szyf, 1984); however, in higher eukaryotes such as vertebrates, only cytosines modified at the 5' position by methylation are found. Methylation, in most cases is sequence specific, i.e., not all cytosines are substrates for methylation. In vertebrates, most of the methylated cytosines are found in the dinucleotide sequence CG (Gruenbaum *et al.*, 1981b); however, some reports indicate the presence of methylated cytosines at the dinucleotide sequences CC, CT, and CA (Woodcock *et al.*, 1987, 1988; Toth *et al.*, 1990; Clark *et al.*, 1995). The significance of methylated cytosines at dinucleotide sequences other than CG is poorly understood; however, a recent report suggests that methylated cytosines other than those residing at the CG dinucleotide sequence are concentrated at origins of replication (Tasheva and Roufa, 1994). This review will focus on CG methylation, because the role of other methylated dinucleotide sequences is poorly understood. Methylated bases are not incorporated into DNA by the regular replication machinery, but the cytosines are covalently modified with a methyl group postrep-

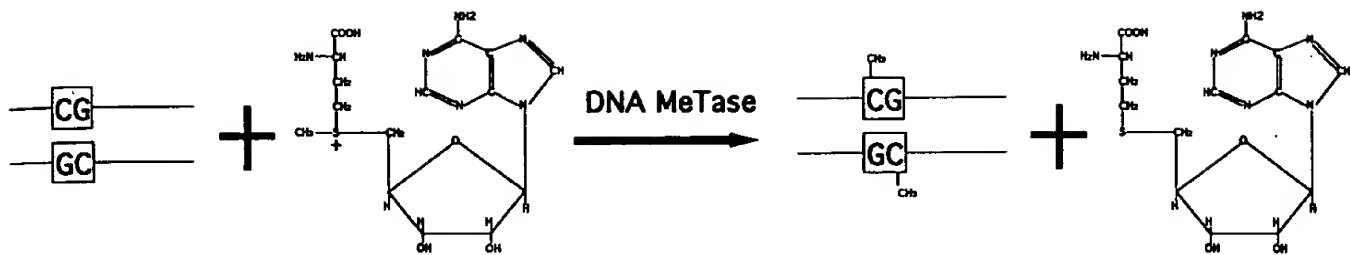


FIGURE 1. The DNA methylation reaction. The modification of DNA is a postreplication process that is catalyzed by a DNA MeTase enzyme. The enzyme recognizes a potential methylation site in the DNA (the dinucleotide sequence CG) and transfers a methyl group (CH₃) from the ubiquitous methyl donor SAM. The reaction products are a methylated CG site in the DNA and SAH.

lication by DNA methyltransferase (MeTase) enzyme(s) (Fig. 1), which, in most cases, recognize the cytosine in its appropriate dinucleotide sequence context (Gruenbaum *et al.*, 1982; Wu and Santi, 1985). The fact that DNA methylation is accomplished by an independent enzymatic machinery provides the genome with additional versatility and provides interesting possibilities for pharmacological interventions (Szyf, 1994).

3. DNA METHYLATION PATTERNS AS EPIGENETIC REGULATORS OF EXPRESSION OF GENOMIC FUNCTIONS

3.1. DNA Methylation Patterns

Although the genetic information is the same in all tissues that constitute a multicellular organism, the expression of functions encoded by the genome varies in different tissues. In some instances, the two alleles of the same gene will be expressed differently in the same cell. For example, X-inactivation involves the inactivation of the allele of a gene that resides on the inactive X chromosome, whereas the other allele of the same gene that resides on the active X chromosome is active (Migeon, 1994). Another striking example is the phenomenon of "parental imprinting," where an allele of a gene that is inherited from one parent is active, whereas the other allele of the same gene that is inherited from the other parent is inactive (Sapienza *et al.*, 1987; Peterson and Sapienza, 1993). One of the fundamental questions in biology is how the same genetic information can encode so many diverse functions in different cells and tissues. The fact that DNA contains a set of modifications that is not encoded in the genetic sequence, but is added covalently to DNA using a different enzymatic machinery, raises the interesting possibility that DNA methylation is a candidate to encode this additional level of information that controls the expression pattern of the genome, "epigenetic information" (Holliday and Pugh, 1975; Holliday, 1990; Razin and Riggs, 1980). Therefore, in the early days of the field, several groups tested the hypothesis that different tissues bear different levels of 5-methylcytosine (Ehrlich *et al.*, 1982; Gama-Sosa *et al.*, 1983a; Gruenbaum *et al.*, 1981b; Razin *et al.*, 1984). All studies concluded that, although some differences exist between the total level of cytosine methylation in different tissues, such as the low level of methylation in

placenta and high levels observed in brain and thymus, these differences *per se* cannot explain the differentiation state of these tissues. Using a modification of the "nearest neighbor assay," which enabled the analysis of the level of methylation of cytosines at a specific dinucleotide sequence (Gruenbaum *et al.*, 1981b), it has been shown that not all CG dinucleotides are methylated in the genome; between 70 and 80% of cytosines residing at the dinucleotide sequence -CG- are methylated in most tissues and cell lines analyzed (Razin *et al.*, 1984). Are these nonmethylated cytosines randomly distributed? The advent of a methylation-sensitive (HpaII) and insensitive (MspI) restriction enzyme pair (Waalwijk and Flavell, 1978a,b; Singer *et al.*, 1979) that recognizes a subset of the CG sequences (CCGG), and the development of Southern blotting techniques that enable the visualization of restriction enzyme cleavage patterns of specific genes, reveal that nonmethylated cytosines are nonrandomly distributed (Waalwijk and Flavell, 1978b). A large number of genes were similarly analyzed in the last decade and a half, and it was found that, in general, genes are nonmethylated at certain CG sites in tissues where they are expressed, but they are methylated in nonexpressing tissues (for a review, see Yisraeli and Szyf, 1984) (Fig. 2). This general paradigm has been supported in the last few years with more sensitive techniques, such as ligation-mediated polymerase chain reaction amplification and genomic sequencing (Pfeifer *et al.*, 1989, 1990a,b), as well as bisulfite mapping, which enable the study of the state of methylation of every cytosine in a specific gene (Frommer *et al.*, 1992; Clark *et al.*, 1994). However, other paradigms of methylation patterns do exist, and the correlation between expression and demethylation of specific sites is not as precise as expected originally (Yisraeli and Szyf, 1984). The significance of this imprecise correlation between gene expression and methylation will be discussed in Section 4. These experiments, however, led to the fundamental concept that DNA methylation might encode its information by forming specific patterns of methylation. Because the default state of a cytosine is methylated and nonmethylated CGs are located at strategic positions, such as 5' of expressed genes or the complete sequence of expressed genes (Yisraeli and Szyf, 1984), it is suggested that hypomethylation of specific genes marks them for expression (Razin and Riggs, 1980). It is the pattern of distribution of nonmethylated cytosines among methylated cytosines that

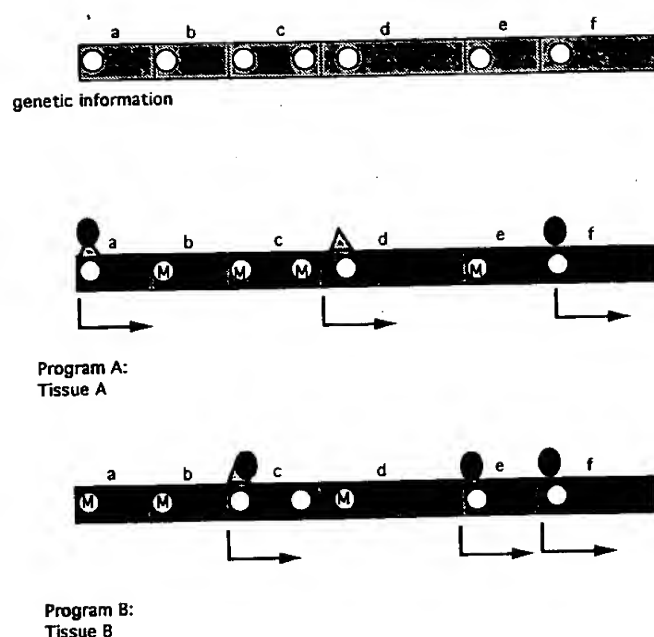


FIGURE 2. DNA methylation patterns. The genome of a vertebrate (first line) bears many potential sites for modification by methylation (open circles). However, a subset of these sites is methylated (indicated by an M in the circle) in different tissues. When one looks at the methylation pattern of different genes (a–e) in different tissues (for example tissues A and B), one observes that they bear a different pattern of methylation. In general, inactive genes are modified, whereas active genes bear sites of methylation that are not modified. It is proposed that both binding of transcription factors to regulatory sites of the genes (indicated by the triangles and ovals), as well as the pattern of methylation, define the state of activity of vertebrate genes.

encodes this information. DNA methylation patterns are, therefore, candidates to encode “epigenetic” information.

3.2. Do DNA Methylation

Patterns Encode Epigenetic Information?

3.2.1. Correlation between hypomethylation and gene expression. What information do methylation patterns encode and how do they encode this information? The fact that genes that are expressed are generally nonmethylated suggests that DNA methylation can repress gene expression. A large number of correlations are established between the state of expression of tissue-specific genes and the state of hypomethylation of specific sites in regulatory regions of genes (Yisraeli and Szyf, 1984). These correlations are extended to X-inactivated genes (Gartler and Riggs, 1983; Pfeifer *et al.*, 1989, 1990a,b; Migeon, 1994). The development of the genomic sequencing technique (Church and Gilbert, 1984) and its ligation-mediated polymerase chain reaction version (Pfeifer *et al.*, 1989) led to the modification of the Maxam and Gilbert sequencing technique (Maxam and Gilbert, 1980), which distinguishes between methylated cytosines and nonmethylated cytosines at the single base level. Using this technique to compare the state of methylation of every cyto-

sine in a CpG island (a region with high density of CG dinucleotide sequences) 5' to the X-linked phosphoglycerate kinase gene, it is shown that the copy of the gene that resides on the inactive X is heavily methylated and the active copy is not (Pfeifer *et al.*, 1990a). These correlations recently have been extended to parentally imprinted genes (Sapienza *et al.*, 1987; Swain *et al.*, 1987) and some genetically inherited genes, such as the fragile X, where the amplification of a trinucleotide repeat is correlated with inactivation of the gene and its hypermethylation (Sutcliffe *et al.*, 1992; Knight *et al.*, 1993). Similar correlations have been observed between retroviral gene expression and DNA methylation and integrated adenovirus gene expression and methylation (Sutter and Doerfler, 1980; Harbers *et al.*, 1981; Kruczek and Doerfler, 1983; Simon *et al.*, 1983). Although the finding that parental imprinting correlates with DNA methylation and that the fragile X syndrome is associated with hypermethylation generated some excitement, these seemingly novel roles for DNA methylation are essentially slight variations on the basic theme suggested by Razin and Riggs in 1980, that hypermethylation correlates with gene repression. The differences in methylation patterns between active and inactive identical genes can explain why the same sequence is expressed in one tissue, but not another, and how two copies of the same gene can be differentially expressed in the same cell, even though they are exposed to the same milieu of transcription factors. However, although the correlation between gene expression and the pattern of methylation is strong, the question remains as to whether or not the pattern of methylation is a consequence of gene expression or a *bona fide* additional level of information? If methylation is a consequence of gene expression, a change in methylation should not alter gene expression and the pattern of methylation should be dependent on the pattern of gene expression. If, on the other hand, the pattern of methylation can control gene expression and encodes epigenetic information, altering the state of methylation of a gene should alter its expression; the pattern of methylation should be determined by factors independent of gene expression. If DNA methylation patterns encode “epigenetic” information, what is the mechanism? Several lines of experiments have been performed by a number of groups to address these questions in the last decade and a half. These experiments are consistent with the hypothesis that DNA methylation encodes an “epigenetic” level of information.

3.2.2. *In vitro* methylated genes are inactive when exogenously introduced into mammalian cells. To test whether or not a change in the state of methylation of a gene can change its pattern of expression, one should be able to alter the state of methylation of a gene without interfering indirectly with its expression. It is technically impossible to specifically change the methylation state of a gene in the cell. However, one can methylate a cloned gene *in vitro* using bacterial enzymes such as *HpaII* (which methylates the sequence CCGG [Singer *et al.*, 1979]) or *SssI* (which methylates all CG dinucleotide sequences [Nur *et al.*, 1985]),

or by *in vitro* synthesizing a hemimethylated substrate on a nonmethylated single-stranded template replacing dCTP with 5-methyl-dCTP (Gruenbaum *et al.*, 1981a; Stein *et al.*, 1982a), introducing it into a cell line and comparing its activity with a mock methylated exogenously introduced gene (Vardimon *et al.*, 1982; Stein *et al.*, 1982b). A long list of experiments utilizing a similar strategy have demonstrated that *in vitro* methylated genes are repressed in mammalian cells. These experiments provided some insight into the possible mechanisms involved in repression of gene expression by methylation. The first question to be addressed by these experiments is whether or not methylation of a specific site is required for gene repression, or whether or not a regional nonsite-specific methylation of sequences in and around the gene is sufficient to silence transcription. The second question to be asked is whether or not methylation inhibits directly the transcription process, or whether or not other proteins that recognize and bind methylated DNA mediate the inhibition of gene expression. In some cases, such as viral promoters (the E2A adenovirus promoter) (Langner *et al.*, 1984) or the proenkephalin gene (Comb and Goodman, 1990), the methylation of specific sites, mainly transcription factor recognition sequences (such as activator protein (AP)-2 in the proenkephalin gene), was sufficient to repress transcription. In other examples, no site specificity was observed and methylation of different sites in the coding region (Yisraeli *et al.*, 1988) or in associated sequences, even juxtaposed plasmid sequences, could mediate repression of different genes (Bryans *et al.*, 1992; Komura *et al.*, 1995). These observations support the conclusion that DNA methylation could interfere with gene expression by at least two different mechanisms. One mechanism involves the inhibition of binding of transcription factors to DNA (Comb and Goodman, 1990; Prendergast *et al.*, 1991). The other mechanism, which is nonsite-specific, involves most probably an inactive chromatin structure around the gene (Kass *et al.*, 1993). These two mechanisms have been supported by biochemical and molecular data, as will be discussed in Section 4.

3.2.3. 5-Azacytidine and antisense to the DNA methyltransferase mRNA induce expression of silenced genes.

Although *in vitro* methylation and transfection experiments support the hypothesis that methylation can repress gene expression, one major criticism of this line of experiments is that exogenously introduced genes might behave differently than endogenous genes. One might argue that *in vitro* methylation alters the manner by which transfected DNA is integrated into the genome and might artificially target the introduced DNA to inactive domains in chromatin. This proposed model for the repression of exogenous genes by methylation might be supported by the fact that in many cases, there is no requirement for methylation of discrete sequences to repress transfected genes. Inhibition of transiently transfected genes is observed only after a chromatin structure has formed, suggesting that methylation suppresses the formation of an active chromatin structure, but has no

effect on transcription *per se* (Kass *et al.*, 1993). One might argue that the results obtained with transient transfections are a consequence of the peculiar process of formation of chromatin on exogenous DNA. To demonstrate that DNA methylation plays a role in controlling endogenous gene expression, one has to be able to inhibit methylation of endogenous genes and measure its effects on gene expression. One of the early tools used in inhibiting DNA methylation was the cytidine analogue 5-azacytidine (5-azaC), which is transformed to a trinucleotide by cellular enzymes and incorporated into the nascent chain of DNA during replication. After it is incorporated into DNA, it covalently binds and inhibits the activity of DNA MeTase, the enzyme that catalyzes the DNA methylation reaction (Wu and Santi, 1985). 5-AzaC treatment results in hypomethylation of DNA. A series of experiments pioneered by Jones' group and repeated by many others showed that many silent genes could be induced by 5-azaC (Constantinides, 1977; reviewed by Jones, 1985). These experiments also suggested that DNA methylation can control cellular differentiation because a number of cell lines could be induced to differentiate with 5-azaC, e.g., Friend erythroleukemia cells (Creusot *et al.*, 1982) and a number of fibroblast lines NIH 3T3 and 10T1/2, which differentiated into muscle, fat, and osteoclast cells (Taylor and Jones, 1979). A series of experiments have suggested that demethylation of a single locus induced by 5-azaC was sufficient to convert 10T1/2 cells into determined myoblasts, leading to the identification of a gene that was proposed to function as a master regulator of myogenic differentiation, *MyoD* (Lassar *et al.*, 1986; Davis *et al.*, 1987).

However, despite the wealth of data generated by this highly effective inhibitor of DNA methylation, some critical questions remain. The main criticism of 5-azaC experiments is the fact that 5-azaC is a nucleoside analog that can inhibit other DNA metabolizing enzymes and might interfere with other DNA-binding functions that are critical for maintaining the state of gene expression. Indeed, 5-azaC has been shown to induce new developmental phenotypes in *Aspergillus*, whose DNA does not contain 5-methylcytosine (Tamame *et al.*, 1983), and a recent report by Jaenisch's group suggests that 5-azaC can act by a mechanism that involves trapping the DNA MeTase by its covalent binding to the 5-azaC residues in DNA, rather than inhibition of DNA methylation (Juttermann *et al.*, 1994). 5-AzaC is mutagenic in bacterial (Call *et al.*, 1986; Lal *et al.*, 1988) yeast, which do not contain 5-methylcytosine in their genome (Zimmermann and Scheel, 1984), and mammalian systems (Amacher and Turner, 1987). It induces DNA damage (Covey *et al.*, 1986) at fragile sites in chromosomes and chromosomal breakage (Lavie *et al.*, 1985; Snyder and Lachmann, 1989; Djalali *et al.*, 1990) even in *Drosophila*, which do not bear 5-methylcytosine in their genome (Katz, 1985; Osgood and Seward, 1989). It is teratogenic in both the mouse (Takeuchi and Takeuchi, 1985; Matsuda, 1990) and the rat (Rosen *et al.*, 1990). The toxic side effects of 5-azaC are consistent with classic nucleoside analogue effects, as exemplified by its rapid antiviral effects, which do not seem

to be mediated by an inhibition of DNA methylation (Bouchard *et al.*, 1990). Many investigators have tried to avoid some of these problems by using concentrations that were below the toxic doses, but it is still very hard to determine whether or not the effects on differentiation are exclusively a consequence of hypomethylation. To address these inherent limitations in 5-azaC, an antisense approach was used to partially inhibit DNA MeTase expression in 10T1/2 cells. These experiments demonstrated that an inhibition of DNA methylation, by expressing a cDNA encoding the DNA MeTase in the antisense orientation in 10T1/2 cells, resulted in conversion of the cells to a myogenic phenotype (Szyf *et al.*, 1992); thus, supporting the hypothesis that DNA methylation controls the state of differentiation of somatic cells.

The experiments by Szyf *et al.* (1992) raise interesting questions regarding the mechanisms by which inhibition of DNA methylation induces differentiation: Does inhibition of DNA MeTase result in demethylation and induction of a gene that induces differentiation, such as *MyoD*? Even though this is the most simple and attractive model to explain induction of differentiation by demethylation, this model implies that *MyoD* is methylated in nonmuscle cells where it is not expressed. However, recent data show that *MyoD* is not methylated in many somatic cell types that do not express the gene (Jones *et al.*, 1990a). However, *MyoD* is specifically methylated in 10T1/2 cells and demethylated following antisense or 5-azaC treatment (Jones *et al.*, 1990b; Szyf *et al.*, 1992). Jones and colleagues have shown that methylation of a CpG island flanking the first exon of *MyoD* occurs during the process of cellular transformation in culture, but the gene is not methylated *in vivo*, suggesting that the methylation of *MyoD* is unique to a cell culture situation and does not reflect the situation *in vivo* (Jones *et al.*, 1990b). Our results with the DNA MeTase antisense 10T1/2 transfectants suggest that other DNA sequences are demethylated before *MyoD* is demethylated and expressed (Szyf *et al.*, 1992). If this model is true, other genes that are methylated in 10T1/2 cells and in embryonal mesenchymal cells "*in vivo*" are induced by their respective DNA demethylation and, in turn, these genes activate *MyoD* and reverse its aberrant methylation pattern. An interesting observation that supports the hypothesis that additional genes are induced by demethylation during myogenesis is that 5-deoxyazacytidine (5-azaC; the deoxy analogue of 5-azaC) potentiates the myogenic activation by *MyoD* of an osteogenic sarcoma line (Chen and Jones, 1990). The hypothesis that two classes of methylation/demethylation events exist, primary methylation sites that play a causal role in controlling gene expression and secondary sites that alter their methylation state in response to changes in gene expression, can reconcile the contradictory data defining the temporal and causal relationship between gene expression and DNA methylation (Fig. 3) (Yisraeli and Szyf, 1984; Szyf, 1991). Most studies focus on the involvement of DNA methylation in regulating gene expression during cellular differentiation, but an alternative hypothesis that should also be considered is that inhibition of methylation of specific sites directly controls the differen-

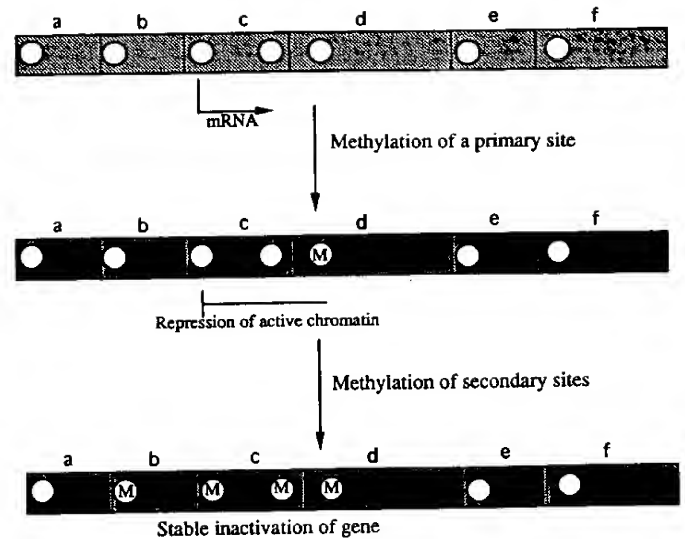


FIGURE 3. Primary and secondary methylation. Methylation (M) of a primary methylatable site (open circle) targets another gene sequence in *cis* for repression. The primary site might be located in the gene itself or associated genes. This can serve as an imprint signal in parentally imprinted genes. The primary site will induce repression of the associated gene by binding a factor that will precipitate in *cis* an inactive chromatin structure. The resulting state of repression and the inactive chromatin structure might target the gene itself for methylation directly or indirectly. This methylation is secondary to the changes in chromatin structure induced by repression of the gene.

tiated state of the cell in a mechanism that does not involve gene expression.

3.3. Parental Imprinting and X-Inactivation: Models for Methylation-Directed Epigenetic Control of Gene Expression

One of the basic questions in biology is how the same genetic information is expressed differently in diverse cells. The remarkable advances in discovering multiple tissue-specific DNA-binding factors and transcription factors as regulators of tissue-specific gene expression has relegated DNA methylation to a secondary role. There are two examples, though, where the presence of different *trans*-acting factors cannot explain differential gene expression: X-inactivation and parental imprinting. Both involve allele-specific gene expression, whereby only one of a pair of identical allelic genes is expressed, although they are exposed to the same *trans*-acting factor milieu (for reviews on X-inactivation, see Gartler and Riggs [1983] and Migeon [1994]; for reviews on parental imprinting, see Peterson and Sapienza [1993] and Efstratiadis [1994]). There must be some "epigenetic" mechanism that marks genetically identical alleles for differential expression. In X-inactivation in females, almost all of the genes located on one of the pair of X chromosomes are inactivated in the trophoblast cells and later in the primitive ectoderm that will develop into the embryo proper (Lyon, 1992). Intensive genetic dissection of this phenomenon has shown that in-

activation of the X chromosome spreads from an inactivation center in *cis* in one of the chromosomal pair, and is controlled by the X-controlling element locus, which maps in the mouse to this center (Lyon, 1992). Riggs (1975) has suggested that methylation of DNA might be the mechanism responsible for inactivation of the X chromosome, where differential methylation is started at the inactivation center and then travels in *cis* along the chromosome. In accordance with this model, several groups have shown that 5-azaC could activate silent X-linked genes (Mohandas *et al.*, 1981; Hansen and Gartler, 1990), and that X-linked genes are methylated on the inactive chromosome and nonmethylated in strategic sites on the active X chromosome (Keith *et al.*, 1986; Pfeifer *et al.*, 1989, 1990a,b; Singer-Sam *et al.*, 1990a). However, two lines of evidence have shed doubt on the hypothesis that DNA methylation plays a primary role in X-inactivation. First, there is evidence indicating that the nontranslatable Xist RNA transcript, which is specifically expressed from the silent X, is responsible for the *cis*-inactivation of the X chromosome (Brown *et al.*, 1991; Kay *et al.*, 1993). Different models have been suggested to explain how an action of a protein(s) progressing in *cis* from the X-inactivation center results in spreading the inactive state throughout the chromosome and why the activity of the *cis*-acting factor is blocked on the other member of the X chromosome pair. This question is far from resolved and is beyond the scope of this review (Lyon, 1992). Second, Lock *et al.* (1987) have shown that differential methylation of the X-inactivated hypoxanthine phosphoribosyl transferase (*hprt*) gene occurs after initiation of X-inactivation. The fact that X-inactivation in marsupials does not seem to be associated with differential DNA methylation is consistent with the model that DNA methylation is not a critical component of the inactivation process. The currently accepted model is that differential methylation as a covalent modification of inactive X-linked genes helps stabilize the inactive state (Lock *et al.*, 1987; Kaslow and Migeon, 1987; Lyon, 1992). This is consistent with the fact that X-inactivation is less stable in marsupials than in eutherian mammals (Lyon, 1992). This model could also be extended to explain the role of methylation in gene expression in general, as discussed in Section 3.3. However, more recent data have provided some reinforcement of the DNA methylation hypothesis. Xist itself exhibits differential methylation that inversely correlates with its state of expression, and differential methylation of Xist alleles precedes the onset of X-inactivation in differentiating mouse embryonal stem cells (Norris *et al.*, 1994). Both Cedar's and Monk's groups have shown that the inactivation of the maternal X chromosome in extraembryonic tissues correlates with the presence of gamete-derived methylation at CG sites 5' to the gene (Ariel *et al.*, 1995; Zuccotti and Monk, 1995). These data point to the possibility that some methylation sites might be the primary signal for X-inactivation.

Parental imprinting is the selective expression of one member of a pair of identical genes based on its parental origin. It has been observed that the maternally and paternally

inherited genomes, despite their almost identical genetic information, do not function equally (Efstratiadis, 1994). A minority of genes that are imprinted are expressed only when inherited from one parent, and the copy inherited from the other parent is silent (Peterson and Sapienza, 1993). The most convincing data for imprinting comes from pronuclear transplantation experiments. Using this technique, embryos that inherited both sets of chromosomes from either maternal or paternal origin were shown to be nonviable (McGrath and Solter, 1984), suggesting that the proper development of a mouse requires both the maternal and paternal genomes. In a few recent years, a number of genes were identified to be expressed monoallelically, based on the parent of origin of the allele (*H19*, insulin-like growth factor II [*IgfII*], insulin-like growth factor II receptor [*IgfIIr*]). It is clear that the genes are imprinted at gametogenesis and the imprint is maintained throughout development, whether the gene is expressed in specific tissues (mouse *IgfII*) or whether it is ubiquitously expressed (human *IgfII*) (reviewed by Efstratiadis, 1994). It is clear that an "epigenetic" mechanism is involved in imprinting. As the imprinting signal has to be maintained throughout rounds of replication during the developmental process, it stands to reason that DNA methylation, which is the only known covalent modification of mammalian DNA, is the imprinting signal. An alternative, but unlikely, hypothesis is that the imprinting signal is a binding protein that is at least a heterodimer (where each of the daughter strands bind to one monomer during replication to maintain the signal) and is tightly bound to the DNA during fertilization (Efstratiadis, 1994). DNA methylation is an attractive hypothesis because it is believed that after a pattern of methylation is established by *de novo* methylation during gametogenesis, it will be maintained during successive replications by semiconservative maintenance methylation (Razin and Riggs, 1980). Thus, the methylation pattern is maintained throughout fertilization and successive rounds of replication. DNA methylation is the only known specific modification of DNA that could be faithfully inherited. As the "epigenetic" information in parental imprinting has to cross the generation barrier, parental imprinting provides the strongest argument for DNA methylation as a carrier of epigenetic information. This is in contradistinction with X-inactivation (except extraembryonic tissues), where the marking of the inactive alleles occurs during development.

Parentally imprinted genes, however, turns out to be as problematic as other previously studied classes of genes when the correlation of methylation and gene expression is carefully analyzed. Although early experiments with parentally imprinted transgenes showed remarkable correlation between "imprinting" and hypermethylation, later experiments have questioned the basic premise that the original pattern of methylation of the imprinted gamete is maintained throughout development. Methylation-imprinted genes do not escape the rounds of demethylation and remethylation that occur during preimplantation and postimplantation stages (Kafri *et al.*, 1992; Razin and Kafri, 1994), and the

pattern of methylation of imprinted genes is established later in development. For example, mouse *H19* gene is hypermethylated in the paternally inherited silent allele, but a developmental analysis of the methylation pattern of the two alleles reveals that only some of these sites are methylated in the sperm, and even this methylation is erased at the morula/blastula stage, reappearing later in development (Bartolomei *et al.*, 1993; Brandeis *et al.*, 1993b). Like tissue-specific genes and X-inactivated genes, parentally imprinted genes present the same mixed signals regarding the question of whether or not DNA methylation is the carrier of "epigenetic" information or a reflection of the state of gene expression. I previously have suggested that a way to reconcile the contradictory data on methylation patterns of imprinted genes is to differentiate between secondary DNA methylation changes and primary imprinting DNA methylation signals (Szyf, 1991) (Fig. 3). The primary methylation signal could be located in an associated regulatory sequence or in *cis* in a separate gene (*H19* and *IgfII*). According to this hypothesis, a primary methylation event occurs in gametogenesis. The pattern of methylation of adjacent sites will be established during development by interaction of the primary methylated or hypomethylated sites with factors that recognize their state of methylation and interpret it (Szyf, 1991). This hypothesis suggests that the interpretation of a primary methylation signal requires a complex set of protein-DNA interactions that, in turn, will affect the methylation state and expression of downstream sequences and genes. Therefore, a simple correlation between a specific methylation pattern of sites and gene expression will be misleading. I suggest that one of the essential limitations of the standard analysis of methylation patterns is the fact that these assays cannot differentiate between primary and secondary methylation events. The model I propose is supported by recently published data. One example is the maternally expressed *IgfIIr* gene (Stoger *et al.*, 1993). The promoter of the *IgfIIr* gene is methylated in the inactive paternal allele, but this methylation is established late in embryogenesis (secondary methylation). An intron 27 kb downstream from the transcription initiation site bears two sites that are methylated in mature oocytes, and they remain so throughout development (primary methylation event). It is suggested that this methylation imprints the active gene by attracting a methylation-dependent activator or inhibiting the binding of a methylation-sensitive repressor (Stoger *et al.*, 1993). Another example of the difference between primary and secondary signals is the reciprocal imprinting of the adjacent *H19* and *IgfII* genes. *IgfII* and *H19* are positioned next to each other at 11p15.5. The maternal *H19* gene is expressed and *IgfII* is silent, whereas the paternal *H19* is repressed and the *IgfII* is active (Bartolomei *et al.*, 1993). It was suggested that, by an unknown mechanism, the expression of *H19* could repress in *cis* the expression of *IgfII*. *H19-IgfII* imprinting can serve as a model for a primary methylation-dependent imprint located in a different gene. A recent report that analyzed the methylation and expression of *H19* and *IgfII* in a number of Wilms tumors (WTs) is also consistent with

this hypothesis. When *H19* expression is detected (maternal allele expressed), *IgfII* expression is monoallelic (maternal allele repressed). *H19* is not methylated on the maternal allele, but *IgfII* is methylated. The methylation of *IgfII* in WTs is a secondary tissue-specific event, because *IgfII* is not methylated at these sites in other tissues, regardless of its expression status (Sasaki *et al.*, 1992). However, in WTs, where the imprinting of *IgfII* is relaxed, the maternal *IgfII* is expressed and associated with hypermethylation of *H19*. Methylation or hypomethylation of *H19* is the primary event that determines the state of expression and secondary methylation pattern of the *IgfII* gene (Taniguchi *et al.*, 1995). Although none of the primary sites of methylation have been determined unequivocally, the experiments listed above demonstrate the concept of an alternating cascade of methylation and changes in gene expression.

Although the correlation between site-specific methylation and imprinting has been confusing, the impact of a general inhibition of DNA methylation by a knockout mutation of the DNA M_{et}ase gene on imprinting is dramatic (Li *et al.*, 1993). In a DNA M_{et}ase null/null background, the transcriptional activity of two imprinted genes, *IgfII* and *IgfIIr*, is extinct, whereas the expression of *H19* is biallelic. The fact that loss of methylation can result in the extinction of expression of the *IgfIIr* gene is consistent with the hypothesis that oocyte-originated methylation of an intronic region in the maternal *IgfIIr* imprints it as the active allele. These experiments, measuring the impact of a general inhibition of methylation on parentally imprinted gene expression, establish a causal relationship between methylation and expression of imprinted genes. However, as is the case with X-inactivation and gene expression (discussed in this section and Section 3.2), these results do not exclude the model in which DNA methylation plays a stabilizing role rather than a primary role in establishing the pattern of gene expression.

3.3.1. Summary. Is DNA methylation the carrier of epigenetic information? This question has been approached by three different strategies: (1) correlation between methylation patterns and expression of tissue-specific genes, house-keeping genes, X-inactivation, and parental imprinting; (2) exogenous expression of *in vitro* methylated genes; (3) inhibition of expression of the DNA M_{et}ase pharmacologically by 5-azaC (Jones, 1985), by antisense expression (Szyf *et al.*, 1992), and by homologous knockout (Li *et al.*, 1992). The experiments using general inhibition of DNA methylation by knockout and antisense provide the strongest evidence that DNA methylation plays a causal role in controlling gene expression, differentiation, parental imprinting, and X-inactivation. The correlative studies of DNA methylation patterns and gene expression have provided us with a perplexing set of observations that are consistent with a causal role, on one hand, and a secondary role, on the other hand. One way of reconciling this paradox is hypothesizing that some methylation patterns can act as primary causal events in gene expression, but other patterns are secondary to gene

expression. What might confound our ability to interpret one isolated point in this complex sequence of events is the fact that the primary methylated sequence might signal activation of a gene (as is the case with the imprinting of *Igf1lr*), and the secondary methylation change will be inversely correlated with the state of expression of a gene (as is the case with many parentally imprinted genes, as well as tissue-specific genes). The primary and secondary signals might be differentially expressed in development. For example, a DNA MeTase knockout does not express both alleles of the *Igf1l* gene (which is paternally expressed in wild-type mice), suggesting that methylation is required for their expression (Li *et al.*, 1993). In contrast, 5-azaC-treated maternally chromosome 7 disomic (MatDi7) cell lines (untreated maternally disomic cells do not naturally express *Igf1l*) express *Igf1l* from the maternal alleles, suggesting that hypomethylation is required for expression of *Igf1l* from the maternal allele (Eversole-Cire *et al.*, 1993). The opposite effect of inhibition of methylation on the pattern of expression of the developing embryo (Li *et al.*, 1993) and a cell line (5-azaC-treated cells) might reflect the fact that, at early stages of development, the primary methylation signal (probably methylation and repression of *H19*, which activates the paternal allele) is dominant but, at later stages, the secondary signal, which maintains the inactive state of the maternal allele, is critical. It should be noted that the genomic location of these signals has not yet been identified, even though the data obtained by general inhibition of DNA methylation suggests that they should exist.

How can methylation act as an inhibitor of gene expression as well as an activator of gene expression? How can methylation both affect and be an effect of gene expression? A previously published hypothesis suggested that the classic concept of linear relationship between DNA methylation and expression (Razin and Riggs, 1980) be replaced with a model describing a circular relationship between DNA methylation and gene expression (Fig. 4). Thus, to understand whether DNA methylation is a cause or effect of gene expression, one has to understand how methylation patterns are formed and how they perform their biological role. These questions are to be discussed in Section 5.

What are the pharmacological and therapeutic implications of DNA methylation? The fact that general inhibitors of DNA methylation can control the "epigenetic" profile of the genome provides us with an interesting pharmacological and therapeutic target. Manipulating the DNA methylation machinery pharmacologically could provide us with the tempting possibility of controlling and altering the profile of gene expression in specific tissues and the whole organism. This could have important therapeutic applications in diseases that involve aberrant programs of gene expression, or in situations where expression of silent genes could be of therapeutic advantage. To fully realize and understand the therapeutic potential of DNA methylation, one has to understand how methylation patterns are regulated, formed, and maintained and how they perform their biological functions.

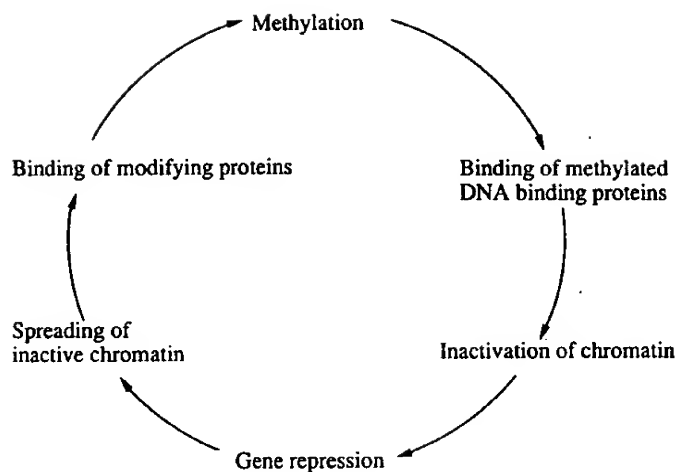


FIGURE 4. Circular relationship between gene repression and DNA methylation. A sequence is targeted for DNA methylation by "modifying proteins." Methylation of DNA results in the precipitation of an inactive chromatin structure that, in turn, causes gene repression. Gene repression can cause the further spreading of an inactive chromatin structure that, in turn, can enhance the propensity of adjacent sequences to become methylated directly. Methylation might be enhanced by the binding of "modifying proteins," which target a sequence for methylation.

3.4. DNA Methylation and Cancer

3.4.1. Oncogenes and tumor suppressors might be regulated by epigenetic mechanisms. A long line of experiments has firmly established that the initiation and progression of cancer involves a change in the genetic program. This process involves an aberrant activation of genes that can promote growth (oncogenes) (Weinberg, 1985) and inhibition of genes that control growth, such as tumor-suppressor genes (Hinds and Weinberg, 1994). Many of these genes have been identified in the last decade. Members of many classes of proteins can turn into oncoproteins (proto-oncogenes) when activated aberrantly, such as tyrosine kinase cell-surface receptors of growth hormones (Schlessinger and Ullrich, 1992), intracellular tyrosine kinases (Schlessinger and Ullrich, 1992), intracellular signaling molecules such as Ras (Lowy and Willumsen, 1993), early immediate transcription factors such as Fos, Myc, and Jun (Herschman, 1991), and translation factors such as the mRNA cap binding factor (eIF-4E) (Lazaris-Karatzas *et al.*, 1990). The downstream effectors of many of these proto-oncogenes are unknown. Another class of genes that was originally identified by positional cloning of the *retinoblastoma* (*Rb*) gene from *Rb* is the tumor suppressors (Levine, 1993; Riley *et al.*, 1994). The loss of function of these genes leads to cancer. The last few years have seen spectacular progress in the identification of tumor-suppressor genes. Whereas some were identified by positional cloning from cancers caused by loss of function of specific genes, such as WT (Hastie, 1994) and neurofibromatosis-1 (Cawthon *et al.*, 1990), many other proteins were identified by an elaborate dissection of the biochemical events controlling the cell cycle, such as the inhibitors of cyclin-dependent kinases p16 and p21 (Elledge

and Harper, 1994). It appears that most tumor suppressors play different roles in controlling the progression of the cell cycle. If DNA methylation, indeed, does encode epigenetic control of gene expression, it may likely be involved in carcinogenesis. Whereas many of the changes that result in the activation of oncogenes or inactivation of tumor-suppressor genes involve stable alterations of the genetic information, such as mutations and rearrangements, it is also reasonable that "epigenetic" alterations, such as DNA methylation, might trigger changes in gene expression that can lead to cancer. Many reports, indeed, have established that cancer cells bear aberrant methylation patterns. However, the results obtained with cancer cells are confusing, seemingly contradictory, and apparently difficult to interpret.

3.4.2. Hypomethylation and cancer. The focus of studies analyzing the correlation between cancer and methylation initially was influenced by the consensus that activation of proto-oncogenes leads to cancer. Because it is believed that hypomethylation could induce gene expression, this will lead to the rather simplistic hypothesis that hypomethylation induces cancer. In accordance with this hypothesis, a series of papers have shown that the genomes of tested cancer cells are widely hypomethylated when either the general level of methylated cytosines is assayed (Gama-Sosa *et al.*, 1983a,b; Feinberg *et al.*, 1988), or when specific genes are looked at with methylation-sensitive restriction enzymes and Southern blot analysis (Feinberg and Vogelstein, 1983; Goelz *et al.*, 1985; Wahlfors *et al.*, 1992). It is clear, however, that hypomethylation is not restricted to oncogenes, and genes that are not involved in cancer progression are also hypomethylated (such as β -globin). This is inconsistent with the model that methylation is a direct reflection of the state of activity of oncogenes or that demethylation is a site-specific mechanism for activation of oncogenes. However, the possibility still exists that the general hypomethylation observed in cancer cells plays a causal role in carcinogenesis, by also hypomethylating and activating oncogenes repressed by methylation, as part of a process that is not site-specific *per se*. There is yet no clear example of a methylation repressed proto-oncogene that is activated by hypomethylation; however, some correlative studies have shown that certain CCGG sites in *ras* and *raf* oncogenes are methylated in mouse liver and are demethylated in liver tumors (Vorce and Goodman, 1989; Ray *et al.*, 1994). An alternative hypothesis is that the general hypomethylation observed in cancer cells is a side effect of the carcinogenic process, rather than an active mechanism that plays a role in the oncogenic process.

The observation that widespread hypomethylation occurs in cancer cells prompted a series of experiments testing the hypothesis as to whether or not inhibition of methylation is involved in oncogenesis. Animals were treated with the DNA M_Tase inhibitor 5-azaC, or were subjected to ethionine replacement (Kanduc *et al.*, 1988), or methyl-deficient diet, resulting in the reduction of cellular S-adenosylmethionine levels (SAM, the donor of methyl groups in the methylation reaction) and hypomethylation of DNA (Wainfan

et al., 1989). Other reports show that 5-azaC, or its deoxyribonucleotide analogue 5-azadC, can induce transformation of oncogene-transfected NIH 3T3 cells (Rimoldi *et al.*, 1991), or transformation of other cell lines, such as C3H 10T1/2 (Hsiao *et al.*, 1985) and BALB/c 3T3 cells (Lubet *et al.*, 1990).

Surprisingly, there are very few reports demonstrating that 5-azaC can induce carcinogenesis in animals. One such report showed that treating male Fischer rats with 5-azaC for 18 months resulted in an increase in the incidence of a variety of tumor types (Carr *et al.*, 1984). Other reports show an increase in rat tracheal epithelial cell transformation (Walker and Nettesheim, 1989). 5-AzaC potentiated chemically-induced liver carcinogenesis in the rat (Rao *et al.*, 1989) and increased carcinogenicity in BALB/c mice (Cavaliere *et al.*, 1987). Although some of the above-mentioned experiments are consistent with the hypothesis that hypomethylation is involved in tumorigenesis, they are not devoid of major problems. 5-AzadC, which is incorporated only into DNA and is, therefore, a specific inhibitor of DNA methylation, did not have tumorigenic effects in rats (Carr *et al.*, 1988), suggesting that the tumorigenic effects of 5-azaC in the rat were not related to hypomethylation of DNA. Methyl-deficient diets can lead to hepatic carcinoma in rats, and it was suggested that this might be caused by the hypomethylation induced by a methyl-deficient diet. However, these diets also induce an increase in the level of DNA M_Tase activity (Christman *et al.*, 1993), which might also be responsible for the carcinogenic effects, as discussed in Section 3.4.3. Methyl-deficient diets might inhibit many other transmethylation reactions, and it is difficult to differentiate these effects from DNA methylation. Further, 5-azaC is a nucleoside analogue that has multiple effects on cellular mechanisms other than DNA methylation, as discussed in Section 3.2.3, and will be discussed in detail in Section 6.3.

Recent data suggesting that pharmacological hypomethylation can lead to cancer should be revisited. First, 5-azaC has been shown to inhibit a number of leukemias in clinical studies (Jehn, 1989; for a review of a recent meeting on 5-azaC in leukemia, see *Leukemia Supplement*, 1993) and *in vitro* (Christman *et al.*, 1983; Gambari *et al.*, 1984; Momparler *et al.*, 1984; Motoji *et al.*, 1985; Attadia, 1993), and has been shown to act as an anticancer agent alone or synergistically with other agents, such as cisplatin (Frost *et al.*, 1990), or as a chimeric compound with arabinofuranosyl-5-azacytosine (Wallace *et al.*, 1989). Second, we recently have shown that 5-azadC and an antisense to the DNA M_Tase mRNA inhibit transformation of an adrenocortical tumor cell line Y1 *in vitro* (MacLeod and Szyf, 1995). 5-AzaC has been shown to inhibit DNA synthesis and cell growth in several human neuroblastoma cell lines (Carpinelli *et al.*, 1993), rat nervous system-derived tumor cell lines (Stark *et al.*, 1989), and a murine melanoma cell line (Cortvriendt *et al.*, 1987). 5-AzadC treatment of mice bearing a germ line mutation leading to adenomatous polyposis coli (APC) neoplasia inhibits the initiation of APC neoplasia, and does not induce carcinogenesis (Laird *et al.*, 1995). Lastly, 5-azaC treatment leads to the suppression of a highly tumorigenic murine cell

line, T984-15, transplanted in BALB/c nude mice (Walker *et al.*, 1987).

3.4.2.1. Summary. Whereas genome-wide hypomethylation is a characteristic trait of many cancer cells, there is yet no data demonstrating selectivity of demethylation to oncogenic sequences. Although inhibition of DNA methylation by 5-azaC has been shown to enhance carcinogenesis *in vivo* and *in vitro*, the data are inconclusive. More recent experiments show that 5-azaC treatment can inhibit cancer. The causal relationship between hypomethylation and cellular transformation is unclear. To address the question of whether hypomethylation is a cause or effect of tumorigenesis, one has to understand the mechanisms leading towards genome-wide hypomethylation in cancer cells.

3.4.3. Hypermethylation and cancer. The emergence of tumor suppressors as an important component of the oncogenic process has led to the realization that inhibition of gene expression can also cause cancerous transformation. Because inactivation of tumor-suppressor activity requires the genetic alteration of both alleles, a rare event by standard mutagenesis, an alternative scenario is allelic homozygosity, and it is suggested that hypermethylation could be involved in inactivation of the second allele (Scrabble *et al.*, 1989). Since then, it has been shown that inhibition of tumor-suppressor activity in a large number of tumors that bear no apparent genetic alterations is associated with hypermethylation, such as Rb tumors (Ohtani-Fujita *et al.*, 1993), WTs (Royer-Pokora and Schneider, 1992), renal carcinoma (Herman *et al.*, 1994), and p16 (Merlo *et al.*, 1995). It has also been suggested that inactivation by hypermethylation of parentally imprinted genes such as *H19* is involved in WT (Taniguchi *et al.*, 1995; Ogawa *et al.*, 1993), and possibly in the somatic overgrowth syndrome Beckwith-Wiedemann (Ogawa *et al.*, 1993; Weksberg *et al.*, 1993). Recently, it has been shown that nickel, a potent carcinogen, silences gene expression by specific chromatin condensation and induces DNA hypermethylation, supporting the hypothesis that hypermethylation and gene silencing is an important mechanism of carcinogenesis (Lee *et al.*, 1995).

3.4.4. DNA hypermethylation in cancer cells shows regional and sequence class specificity. In distinction from DNA hypomethylation, which appears to be genome wide (Feinberg *et al.*, 1988), three classes of genomic sequences have been shown to be specifically hypermethylated in cancer cells: (1) tumor-suppressor genes, as described in Section 3.4.3; (2) CpG island sequences; and (3) tissue-specific genes. Baylin *et al.* (1991) has suggested that regional hypermethylation is associated with progression of cancer, as documented in specific loci in a number of cancer cell types by Baylin's group (de Bustros *et al.*, 1988). For example, abnormal methylation of the calcitonin gene marks progression of chronic myelogenous leukemia (Nelkin *et al.*, 1991). Makos *et al.* (1992) have shown that distinct regional hypermethylation patterns occur on chromosome 17p in human lung and colon cancer DNA. Interestingly, hypermethylation occurs in a region

that is frequently reduced to homozygosity in both tumor types, suggesting that this region bears genes whose inactivation is critical for the progression of cancer (Makos *et al.*, 1992). Recently, a new candidate tumor-suppressor gene, which is activated by p53, *HIC-1* (hypermethylated in cancer-1), was cloned by molecular analysis of the hypermethylated region in chromosome 17p13.3. This gene is ubiquitously expressed in normal tissues, but is underexpressed in different tumor cells where it is hypermethylated (Makos-Wales *et al.*, 1995). Hypermethylation in cancer cells, therefore, appears to demonstrate some specificity by being targeted to specific chromosomal loci and specific sequences. One class of sequences that appears to be targeted for hypermethylation in cancer cell lines is the CpG-rich island sequences (Antequera *et al.*, 1990), which are usually found in the 5' region of housekeeping genes and are hypomethylated in most normal somatic tissues (Bird *et al.*, 1985). Jones *et al.* (1990a,b) have shown that hypermethylation of the *MyoD* CpG island occurs in transformed cell lines *ex vivo*, and proceeds progressively during cellular transformation (Rideout *et al.*, 1994). Abnormal methylation of CpG island sequences on chromosomes 11p and 3p has also been described by Baylin *et al.* (1986) and de Bustros *et al.* (1988) in human lung tumors. Hypermethylation of CpG islands is associated with oncogene-induced transformation of human bronchial epithelial cells (Vertino *et al.*, 1993). Another clinically and pharmacologically relevant example is the methylation of CpG islands of the estrogen receptor in human breast cancer cells, which is associated with hormone resistance (Ottaviano *et al.*, 1994). One additional class of genes that has been shown to undergo specific hypermethylation is tissue-specific genes that characterize the differentiated state of the normal parental cell and are inactivated in the dedifferentiated cancer cell type. One example is the adrenal-specific 21-hydroxylase gene (*C21*), which is hypomethylated and expressed in the adrenal cortex, but is hypermethylated and inactivated in the adrenocortical carcinoma cell line Y1 (Szyf *et al.*, 1989, 1990a). When an exogenous *C21* is introduced into Y1 cells, it is specifically *de novo* methylated, suggesting that the cancer cell maintains the capacity to specifically recognize this gene and target it for *de novo* methylation (Szyf *et al.*, 1989).

What is the mechanism responsible for regional hypermethylation in cancer cells and what is its role in carcinogenesis? A possible mechanism for hypermethylation of DNA in cancer cells is the hyperactivation of the DNA MeTase observed in many cancer cells (Kautiainen and Jones, 1986; el-Deiry *et al.*, 1991). What is the mechanism responsible for increased DNA MeTase activity in cancer cells? What is its possible role? These questions will be discussed in Section 5.11.

3.4.5. Models on the role of DNA methylation in carcinogenesis. What role does DNA methylation play in carcinogenesis? The possible models have to take account of both the hypomethylation and hypermethylation observed in cancer cells. As discussed in Section 3.4.2, hypomethylation was suggested to play a role in activating tumor-

promoting genes (Counts and Goodman, 1994). The implication of this is that hypomethylating agents are carcinogenic. However, as we discussed in Section 3.4.4, the focus has turned to hypermethylation in recent years. Previously, Baylin *et al.* (1991) has suggested that an imbalance of DNA methylation involving widespread hypomethylation and regional hypermethylation results in abnormalities of chromatin organization. This, in turn, can lead to inactivation of genes, predisposition to mutations, and allelic deletions (Baylin, 1991). An alternative hypothesis that has been suggested by Jones and colleagues (1992) is that methylated cytosines are mutation hot spots, because methylated cytosines can be transformed by spontaneous or enzyme-catalyzed deamination into thymidine which, in turn, following replication, will result in mutation of the original C-G bp into a T-A pair. It is suggested that 30–40% of all human germline point mutations occur at methylated CpG sequences. Using direct genomic sequencing, Jones and colleagues have shown that three sites, which are known to be hotspots for mutations in the p53 gene, were found to be methylated in the target human tissue examined (Rideout *et al.*, 1990). Hypermethylation can result in an increase in the number of deaminated CpGs that might strain the mismatch repair machinery normally responsible for removing G-T mismatches (Rideout *et al.*, 1990). Another exciting and surprising result by Jones' group that provides a biochemical basis for the proposed increase in deamination of methylated cytosines in cancer shows that the DNA MeTase enzyme, itself, might catalyze the deamination of methylated cytosines (Shen *et al.*, 1992). Although these experiments used bacterial *HpaII* DNA MeTase, it is reasonable to extrapolate from these experiments to the mammalian enzyme because of the extensive structural homology between the active sites of bacterial and mammalian cytosine DNA MeTases (Cheng *et al.*, 1993; Klimasauskas *et al.*, 1994; Kumar *et al.*, 1994). If DNA MeTase can catalyze the deamination of 5-methylcytosine, in addition to methylation of cytosines, the observed increase in DNA MeTase activity in cancer cells (Kautiainen and Jones, 1986; el-Deiry *et al.*, 1991) can result in an increase in the rate of C:T transitions (Shen *et al.*, 1992; Laird and Jaenisch, 1994).

Does hypermethylation play a causal role in cellular transformation or is it just involved in progression of cancer? An important question is whether hypermethylation of DNA is a component of an oncogenic program, or whether or not it is selected from a random occurrence of aberrant methylation events? If hypermethylation observed in cancer is of pharmacological and therapeutic relevance, its effects should be reversible. On the other hand, if the important consequence of hypermethylation is an increase in the rate of mutagenesis, then the damage incurred by hypermethylation should not be reversible.

3.4.5.1. Summary. Although aberrant methylation patterns consistently are associated with cancer, the directions that these changes take are perplexing. Both hypermethylation of specific chromosomal regions and sequences, as

well as genome-wide hypomethylation, have been observed. How can these two processes coexist and how are they involved in the cancer process? The range of changes in methylation occurring in cancer is inconsistent with a site-specific process that is responsible for, or is a consequence of, the activation of a discrete set of genes. However, some specificity is observed in the hypermethylation of specific loci, CpG-rich islands and tumor-suppressor genes. The broad alterations in methylation patterns could reflect a simple mechanism for bringing about a change in methylation patterns of specific sets of genes, by a general activation or inhibition of DNA methylation or demethylation. Alternatively, the general changes in methylation are important because they are involved in a genome-wide change in chromatin or other genomic parameters. The remaining questions are whether or not sequence-specific hypermethylation and genome-wide demethylation are active components of the transformation program and whether or not reversal of DNA hypermethylation can result in inhibition of tumorigenesis. Obviously, this might have very interesting therapeutic implications. To address these questions, and to design strategies to inhibit cancer-related DNA hypermethylation, one has to understand the mechanisms responsible for hypermethylation in cancer cells. This review will propose a model on the role of DNA methylation in cancer based on a dissection of the mechanisms involved in generating and maintaining DNA methylation patterns.

3.5. Methylation and Expression of Foreign Genes

One of the therapeutic challenges of the next decade will be to utilize the potential of gene therapy. An important delivery concept that is currently used in different gene therapy experiments is the utilization of viral vectors. Three classes of viruses are in current use: retroviruses (Ledley *et al.*, 1987), adenoviruses (Hermonat and Muzyczka, 1984), and herpes viruses (Davar *et al.*, 1992). However, a long line of evidence suggests that DNA methylation is involved in the inactivation of virally introduced genes *in vivo*. Doerfler (1991) has suggested that DNA methylation might act as a general mechanism of inactivation of exogenous DNA and protection of vertebrate genomes against intrusion by foreign genetic material. Doerfler's group has shown that, upon integration, *de novo* methylation spreads from the center of the integrated collinear viral DNA (Orend *et al.*, 1991). Methylation of specific sites in the adenoviral promoters results in inactivation of these promoters (Kruczek and Doerfler, 1982, 1983). Similarly, it has been shown that Moloney murine leukemia virus-long terminal repeat (MoMuLV-LTR) is inactive in embryonic stem cells and embryonic carcinoma cells, and this inactivation is accompanied by *de novo* methylation of the proviral sequences (Harbers *et al.*, 1981; Jahner and Jaenisch, 1985). Recent attempts at introducing different genes into murine hematopoietic cells encountered major problems with long-term expression from the MoMuLV-LTR (Williams *et al.*, 1986; Kaleko *et al.*, 1990). A high rate of expression failure was documented to be associated with

methylation in the proviral LTR (Challita and Kohn, 1994). Herpes virus, which is used to deliver genes in the CNS, also undergoes *de novo* methylation in mammalian cells (Barletta and Greer, 1992). Whereas it has not yet been shown that methylation of the herpes virus is responsible for the repression of its expression *in vivo*, it is clear that expression of herpes virus promoters can be inhibited by methylation. For example, the herpes thymidine kinase promoter is inhibited by methylation, as has been documented in a number of studies (Keshet *et al.*, 1985; Graessmann and Graessmann, 1988; Levine *et al.*, 1991). The inhibition of foreign gene expression by methylation has important therapeutic and pharmacological implications, and inhibition of the methylation of exogenous sequences might enhance gene therapy significantly. To develop effective inhibitors of viral and exogenous DNA methylation, we need to understand the mechanisms responsible for methylation, as will be discussed in the following section.

3.6. DNA Methylation and Replication

Most of the functions that we discuss in this review pertain to different facets of regulation of gene expression. However, it is obvious that the genome has other functions, such as replication, repair, and recombination. DNA methylation is a candidate to control these functions, as well. The role of DNA methylation in controlling replication of *Escherichia coli* genomes is very well established. In *E. coli*, the state of methylation of the origin of replication regulates initiation of DNA synthesis (Messer *et al.*, 1985; Smith *et al.*, 1985; Boye and Lobner-Olesen, 1990). After replication of *E. coli* is initiated, there is a very short lag between initiation of synthesis of the nascent strand of DNA and its methylation by the Dam DNA MeTase (Szyf *et al.*, 1984). The origin of replication is sequestered by the membrane during replication and remains hemimethylated because it is inaccessible to the Dam DNA MeTase (Campbell and Kleckner, 1990). The replication-dependent state of methylation (hemimethylation) is maintained because the level of the Dam methylase in *E. coli* cells is limiting (Szyf *et al.*, 1984). The hemimethylated state of the origin signals that replication is not completed, and new initiations are inhibited (Russell and Zinder, 1987). After replication is complete, the origin is methylated and a new round of replication is initiated (Bakker and Smith, 1989). The hypothesis that DNA methylation regulates DNA replication also in eukaryotes is supported by recent experiments, showing that in *Neurospora crassa*, methylation-deficiency mutants lead to postreplicative chromosomal anomalies (Foss *et al.*, 1993). 5-AzaC treatment can also result in alteration of cell-cycle kinetics parameters, further supporting a potential role for methylation in controlling replication (Poot *et al.*, 1990). The challenge of preventing multiple origins from refiring during the cell cycle of a eukaryotic cell is far greater than the one facing *E. coli*. It stands to reason, therefore, that eukaryotic cells have developed parallel mechanisms. Densely methylated sequence islands have been observed in two Chinese ham-

ster replication origins (*SI4* and dihydrofolate reductase) (Tasheva and Roufa, 1994). All dCs are methylated in these sequences when the cells are cycling, but not when the cells are arrested (Tasheva and Roufa, 1994). The fact that CpA, CpC, and CpT sites are specifically methylated in the origin is surprising because it is generally accepted that DNA methylation in vertebrates occurs mainly at the CpG dinucleotide sequences, as discussed in Section 2. This also raises interesting questions as to the biochemical machinery responsible for this methylation. Is there a specific enzyme methylating CpC, CpT, and CpA sequences? Is this enzyme cell cycle regulated? The paper by Tasheva and Roufa (1994) proposes a biological role for methylation occurring in sequences other than CpG. The authors suggested three possible functions for the densely methylated sequence islands in mammalian chromosomal origins of replication: association with the nuclear matrix, licensing of activation of particular replication origins during specific developmental programs, or molecular signals to mark previously replicated origins (Tasheva and Roufa, 1994).

3.6.1. Summary. Two strategies have been employed to probe the potential role of DNA methylation. The first approach correlates patterns of gene expression with site-specific methylation patterns. This approach has been applied to differentiated tissue-specific genes, parentally imprinted genes, X-linked genes, viruses, oncogenes, and tumor-suppressor genes. These studies have established excellent correlations in many cases but, in a few other examples, no correlation exists between a site-specific pattern of methylation and expression. In certain instances, it is obvious that the pattern of methylation is a consequence of a change in gene expression (Benvenisty *et al.*, 1985). The fact that simple correlations are not universally applicable has confused the field. Even when excellent correlations are found, the question still arises as to whether changes in DNA methylation are a cause or consequence of gene expression? The other approach is to test the effects of inhibition of DNA methylation on functions. This approach has been more convincing in establishing a causal relationship between the above-mentioned examples of differential gene expression and DNA methylation. Quite often, the DNA methylase inhibitor 5-azaC has been used. This approach, which carries the risk of confounding side effects, has been replaced by homologous knockouts and antisense methodologies. The fact that general inhibition of DNA methylation alters differential gene expression patterns, and correlations with specific DNA methylation sites are not always obvious, prompted us to suggest the existence of primary and secondary site-specific methylation events. It is still unclear how to differentiate between primary and secondary methylation sites. If methylation plays such an important role in controlling biological functions, it is of therapeutic and pharmacological importance to be able to alter gene expression patterns by altering DNA methylation. To answer the question of whether DNA methylation is a result of gene expression or its cause, we need to understand how a DNA meth-

ylation pattern is generated. If changing a DNA methylation pattern is of pharmacological advantage, this could be done only when we understand the cellular controls over DNA methylation patterns.

4. MECHANISMS OF ACTION OF DNA METHYLATION PATTERNS

4.1. *Methylated DNA is*

Packaged in Inactive Chromatin

How do methylation patterns perform their proposed functions? It is well established that a gene could be found in either a repressed, derepressed, or activated state. The default position of a gene is in a repressed state, and this state is maintained by the packaging of DNA into nucleosomes in which the DNA is wrapped twice around a core histone octamer (a structure consisting of two copies each of the core histones, H2A, H2B, H3 and H4), and there is one copy of the linker histone H1, which is considered to stabilize the repressed state of the gene (reviewed by Paranjape *et al.*, 1994). The repressed state of the DNA is distinguished by its resistance to DNase digestion. In the derepressed state, the chromatin is unfolded and the template is accessible to transcription factors. In the activated state, transcription factors interact with the gene and activate the basal transcription machinery (Paranjape *et al.*, 1994). Some transcription factors can interact with chromatin and activate a gene by reconfiguring the chromatin, such as the interaction of the glucocorticoid receptor with the mammary tumor virus promoter (Richard-Foy and Hager, 1987). Others interact with the naked DNA during replication and prevent the reassociation of, or cause repositioning of, nucleosomes at the promoter sequences (Paranjape *et al.*, 1994). A ubiquitous factor that can activate the chromatin structure of many genes has been identified in *Drosophila* and is named GAGA (Biggin *et al.*, 1988; Tsukiyama *et al.*, 1994). Because methylated DNA generally is found in inactive chromatin (Razin and Cedar, 1977), DNA methylation might perform its biological role by determining or stabilizing a repressed state. However, an alternative explanation is that DNA methylation inhibits transcription by other mechanisms, and that the repressed chromatin structure is a consequence of repressed transcription. More recent experiments lend support to the hypothesis that DNA methylation can directly mediate the formation of an inactive chromatin structure, because exogenous *in vitro* methylated DNA forms an inactive chromatin structure in mammalian cells when introduced by DNA-mediated gene transfer (Keshet *et al.*, 1986).

4.2. *Methylation Interferes with Binding of Transcription Factors to Their Cognate Sequence*

The discovery of *cis*-acting sequences controlling transcription (Gillies *et al.*, 1983) prompted the hypothesis that DNA methylation inhibits transcription by inhibiting the interaction between transcription factors and methylated *cis*-acting elements. This theory has been validated by the

identification of a number of transcription factors that are inhibited by methylation, such as the binding of AP-2 to the adenoviral E₂A promoter (Langner *et al.*, 1984; Hermann and Doerfler, 1991), the inhibition of the interaction of transcription factors with the tyrosine amino transferase promoter when it is methylated (Becker *et al.*, 1987), the inhibition of interaction of Myc/Max with their cognate sequence when it is methylated (Prendergast and Ziff, 1991; Prendergast *et al.*, 1991), and the inhibition of binding of the cyclic AMP-dependent activator CREB to its cognate site when it is methylated (Comb and Goodman, 1990). However, other experiments suggested that inhibition of binding of transcription factors is not the only or the main mechanism by which methylation inhibits gene expression. For example, the binding of transcription factors to the promoter of the tyrosine amino transferase gene *in vivo* is inhibited by factors other than DNA methylation (Weih *et al.*, 1991).

4.3. *Methylated-DNA Binding Proteins Mediate Gene Repression by Methylation*

To identify *cis*-acting sequences that are sensitive to methylation, transient transfection assays were performed with site-specific *in vitro* methylated DNA. One of the surprising conclusions of these studies is that methylation at different regions in the gene or outside the gene can inhibit transcription without a requirement for methylation of a specific site(s) (Keshet *et al.*, 1985; Yisraeli *et al.*, 1988; Bryans *et al.*, 1992; Komura *et al.*, 1995). These experiments suggested that DNA methylation inhibits transcription by a mechanism other than direct inhibition of interaction of transcription factors with specific sites in the promoter. One possible mechanism is that DNA methylation precipitates and stabilizes an inactive chromatin structure, as suggested before (see Section 4.1) and, therefore, is not dependent on methylation of a specific site. How does DNA methylation stabilize an inactive state of chromatin? One possible mechanism involves the binding of methylated-DNA binding proteins (MDBPs) that precipitate the formation of an inactive chromatin structure. Two such candidate proteins have been identified: methylated CpG binding protein (MeCP1) (Boyes and Bird, 1991) and MeCP2 (Lewis *et al.*, 1992). The presence of these proteins is associated with a chromatin structure that is inaccessible to nucleases such as *MspI* (Antequera *et al.*, 1990). It has been suggested that the binding of MeCP1 to DNA is dependent on the general density of methylated CpG rather than methylation of specific sites, and that strong enhancers can override the effects of MeCP1 (Boyes and Bird, 1992; Hsieh, 1994). This observation is in accordance with earlier work by Dorfler's group showing that expression of the adenoviral protein E1A can reactivate the methylation-repressed E₂A adenoviral promoter (Weisshaar *et al.*, 1988). How do strong enhancers override repression of gene expression mediated by DNA methylation? Perhaps by interacting with inactive chromatin and derepressing it (Paranjape *et al.*, 1994). Alternatively, binding of highly active

transcription factors to enhancer sequences will prevent the formation of an inactive chromatin structure by MeCP1. The data to date, which are based on transient transfection experiments, do not allow one to differentiate between these alternative models.

Binding of methylated DNA by MeCP1 does not require methylation of a specific sequence. However, the formation of an inactive chromatin structure will be more critical at the transcription initiation domain, where the chromatin structure determines the accessibility to the transcription machinery. In accordance with this hypothesis, Levine *et al.* (1992) have shown that methylation at sites flanking the TATA box suppresses mouse metallothionein promoter transcription more efficiently than methylation at a downstream site, suggesting that methylation inhibits the formation of a preinitiation complex. Methylation has no effect on binding of TATA or transcription factors *in vitro* in the absence of chromatin, suggesting that methylation cannot inhibit the transcription machinery *per se*. Therefore, once a transcription preinitiation complex is formed, methylation cannot inhibit it. Similarly, it is well demonstrated that chromatin inhibits the formation of a preinitiation complex and that strong transcription factors can override this inhibition. However, after a preinitiation complex is formed, it is not inhibited by chromatin (Croston and Kadonaga, 1993). Although there is no direct evidence that the binding of MeCP1 is occurring *in vivo* and that its binding is precipitating the inactive chromatin structure, the data presented by Levine *et al.* (1992) is consistent with this model. The lack of purified MeCP1, thus far, has prevented testing the hypothesis that binding of MeCP1 to methylated DNA precipitates the formation of an inactive chromatin in an *in vitro* chromatin reconstitution assay (Paranjape *et al.*, 1994). If methylation inhibits the formation of a preinitiation complex, why does methylation of regions of the gene outside the preinitiation domain affect transcription? Methylation of coding sequences rather than regulatory sequences have been reported to inhibit expression of a chimeric chloramphenicol acetyl transferase construct (Komura *et al.*, 1995). One possible mechanism is that an inactive chromatin conformation can migrate from a focus toward adjacent regions, as has been previously shown (Kass *et al.*, 1993). If this model is true, it can explain the general and imprecise characteristics of DNA methylation patterns that were baffling the field for a long time. The general state of methylation and the density of methylated sites is more critical than the state of methylation of particular sites.

An alternative mechanism by which DNA methylation can alter chromatin structures is by directly modulating the interactions of histone proteins and DNA, or the conformation of histone-bound DNA. Because H1 histone is bound to internucleosomal DNA and is believed to play an important role in establishment of condensed and repressed chromatin (Long *et al.*, 1979; Paranjape *et al.*, 1994), it is a good candidate to be directly affected by DNA methylation. Although it is still unclear whether or not the affinity of H1 to methylated DNA is different than its affinity to non-

methylated DNA (Johnson *et al.*, 1995), Higurashi and Cole (1991) have shown that the combination of H1 histone binding and methylation could alter the sensitivity of DNA to *MspI*, suggesting that methylated DNA bound by H1 is found in a different conformation than nonmethylated DNA. Further, Levine *et al.* (1993) have shown preferential binding of histone H1 to methylated templates. In accordance with this model, Levine *et al.* (1993) and, more recently, Johnson *et al.* (1995) have shown that histone H1 preferentially inhibits transcription from methylated templates. It is still unclear whether or not MDBPs such as MeCP1 are essential for establishing a methylation-directed repressed state of a gene, or whether the interactions of histone H1 with methylated DNA are sufficient to explain the inactive and *MspI*-resistant chromatin structure on methylated CpG islands observed by Antequera *et al.* (1990). Future experiments will be required to determine whether or not MeCP1 is a ubiquitous mediator of repression by methylation, whether or not it is only limited to certain classes of sequences, or whether or not it plays a critical role in establishing repressed states *in vivo*.

4.4. Sequence Specific Methylated-DNA Binding Proteins

The idea that methylation plays its biological role by attracting proteins that specifically recognize methylated DNA sequences has been reinforced by the identification of proteins that recognize specific sequences only in their methylated state. These proteins differ from the previously mentioned general nonsite-selective proteins, such as MeCP1 and MeCP2, by recognizing specific methylated sequences. The first example of this class of proteins is MDBP (Huang *et al.*, 1984; Ehrlich and Ehrlich, 1993). However, it is still unclear whether or not the biological role of MDBP *in vivo* is to bind methylated sequences because the same protein can bind to a related sequence (the X box in promoters of major histocompatibility Class II genes) whether or not it is methylated (Zhang *et al.*, 1993). Several other proteins that were thought to bind preferentially to specific sequences when they are methylated were discovered: MDBP-2 is an avian 40 kDa protein that binds preferentially to a methylated sequence in the *vitellogenin II* promoter (Pawlak *et al.*, 1991) and an Sp-1-like binding protein (Jane *et al.*, 1993; Sengupta *et al.*, 1994) that shows enhanced binding to the stage selector element in the γ -globin promoter when it is methylated. Human methylated DNA binding protein binds Sp-1-like elements in the human immunodeficiency virus-1 LTR when they are hemimethylated in a strand-specific manner (Joel *et al.*, 1993). One should apply caution before recognizing a protein as a site-specific-MDBP. MDBP-2, originally shown to interact with a specific methylated sequence in the *vitellogenin II* promoter and inhibit its *in vitro* transcription (Pawlak *et al.*, 1991), later was shown to be a nonsite-selective binding MDBP homologous to H-1 (Jost and Hofsteenge, 1992), and may play a role similar to MeCP1 and 2. Recently, Bruhat and Jost (1995) have shown that the preferential binding of MDBP-2 to methylated DNA

is mediated by phosphoserine residues in the protein, and that the state of phosphorylation is hormonally regulated. It will be interesting to know whether or not this is a general mechanism of regulation of MDBP activity. The role of other putative site-specific proteins is still unclear. These proteins might play a role similar to MeCP1, but in a more precise and limited manner; alternatively, they might compete for binding with a positive transcription factor, or they might represent mediators of functions of DNA methylation other than gene expression.

4.5. Summary

In summary, DNA methylation plays its biological role by either inhibiting or enhancing the binding of DNA-binding proteins. In some instances, methylation of specific sites is critical, such as when methylation inhibits binding of a specific transcription factor or when methylation enhances the binding affinity of a specific transcription factor. However, it appears that methylation can play a more general role in repressing gene expression, by either altering the conformation of histone H1 bound chromatin or by attracting the binding of ubiquitous methylated-DNA-binding factors,

which, in turn, can precipitate an inactive chromatin structure and fix a gene in a repressed state. According to this model, DNA methylation will not inhibit the expression of a gene that is in the active state and has already formed a preinitiation complex.

5. HOW ARE DNA METHYLATION PATTERNS FORMED, MAINTAINED AND ALTERED?

5.1. Understanding How DNA

Methylation Patterns are Formed Is

Critical for Understanding Their Biological Role

The correlation between gene expression and DNA methylation begs the question of whether DNA methylation patterns are mere mirror images of the state of gene expression or are generated by an independent process? To answer this question, one has to understand how DNA methylation patterns are formed (Fig. 5).

5.2. The DNA Methyltransferase Enzyme

The enzyme that catalyzes the DNA methylation reaction is the DNA MeTase, a 190 kDa protein that is composed of a number of interesting motifs and consists of a catalytic

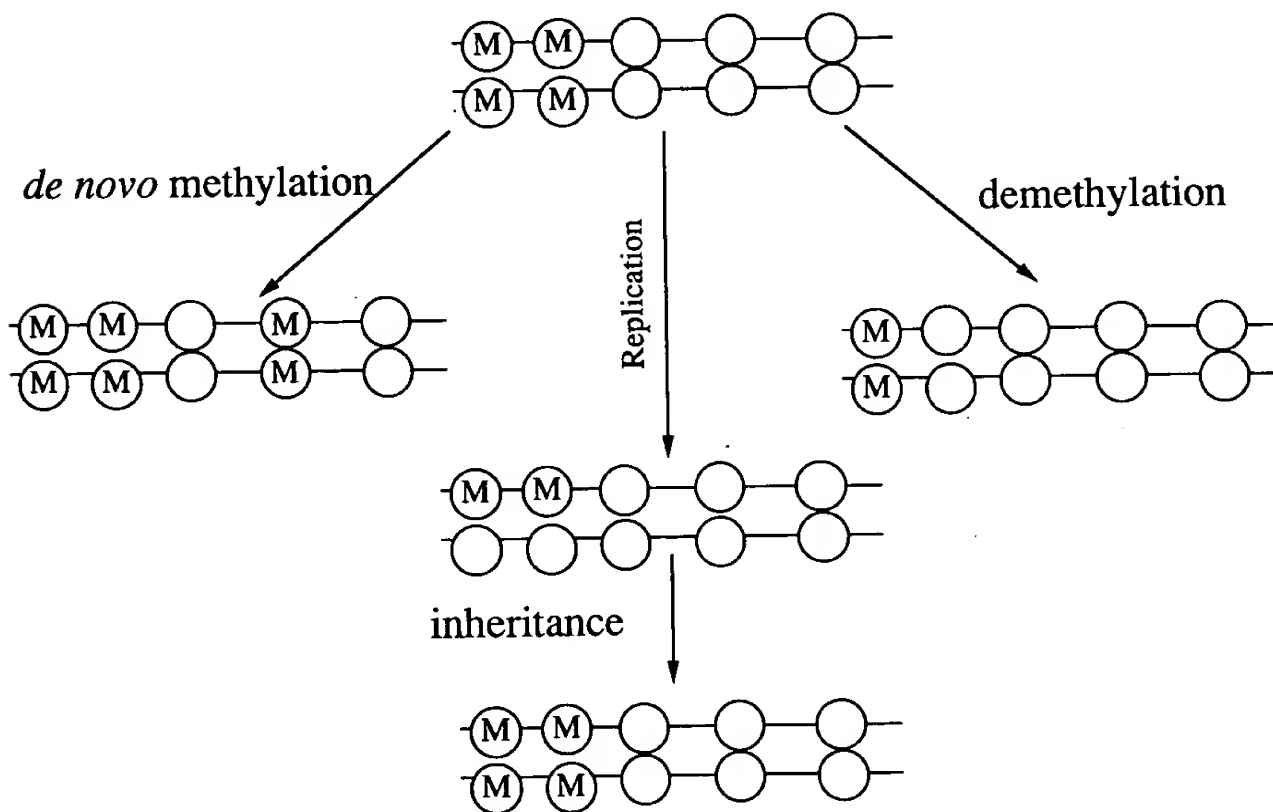


FIGURE 5. Establishment and maintenance of DNA methylation patterns. The establishment of DNA methylation patterns involves a number of processes. Left, *de novo* methylation: when a potential methylatable site (indicated as a circle) that is not methylated on both strands of the DNA undergoes methylation (indicated as M in the open circles). Center, Maintenance methylation: when a site that is methylated is replicated during DNA replication or repair. The new DNA molecule is composed of a parental methylated strand and a nascent nonmethylated strand. Maintenance methylation will introduce methyl groups to the nascent nonmethylatable sites that reside parallel to methylated sites on the parental strand. Thus, the pattern of methylation is replicated. Right, Demethylation: when a methyl group (M) is removed from methylated CpGs.

domain that is homologous to other cytosine DNA MeTases. It has a zinc finger DNA binding domain (Bestor *et al.*, 1988) and a nuclear targeting domain that mediates the association of the DNA MeTase with the replication complex or the "replication factories" (Leonhardt *et al.*, 1992). A mechanism of action of cytosine DNA MeTases suggested by Wu and Santi (1985) recently has been supported by the resolution of the crystal structure of the bacterial *HhaI* MeTase (Cheng *et al.*, 1993). This model predicts that the first step of DNA methylation involves the generation of a covalent intermediate between the enzyme and the carbon at the 6th position of the cytosine base. The transfer of a methyl group to the carbon at the 5th position disrupts the covalent bond and releases the enzyme. Understanding the mechanism of action of the enzyme is critical for the design of inhibitors of DNA MeTase. The model by Wu and Santi (1985) predicted that a fluoro group substitution at the 5th position, which inhibits the transfer of methyl group to cytosine, will also result in stabilizing the covalent intermediate between the enzyme and cytosine in DNA. This prediction has been verified by a large number of experiments, and 5-fluorocytosine-modified DNA is used as a mechanism-based inhibitor of DNA MeTase (Osterman *et al.*, 1988; Chen *et al.*, 1991; Cheng *et al.*, 1993). This is similar to the mechanism of action proposed for the DNA methylation inhibitor 5-azaC, which also forms, after it is incorporated into DNA, a stable covalent bond with the DNA MeTase (Santi *et al.*, 1984). Although mechanism-based suicide inhibitors are very efficient inhibitors of DNA MeTase, they, in turn, will cause trapping of the enzyme to the modified DNA. Trapping of a large number of DNA MeTase molecules to DNA might disrupt DNA function and result in toxicity (Juttermann *et al.*, 1994). Thus, the biological consequences of these inhibitors might not be related to DNA methylation. This potential toxicity might limit the use of such inhibitors in therapy aimed at inhibition of DNA MeTase.

To date, only one enzyme has been shown to be involved in the methylation of mammalian genomes. This enzyme has been shown to catalyze the methylation of both DNA molecules that are methylated on one strand (maintenance methylation), as well as nonmethylated DNA (*de novo* methylation) (Bestor and Ingram, 1983; Bestor *et al.*, 1988) (Fig. 5). Earlier reports, however, demonstrated that the mammalian MeTase is more efficient at methylating hemimethylated strands (Gruenbaum *et al.*, 1982). The cDNA of both the mouse (Bestor *et al.*, 1988) and human DNA MeTase (Yen *et al.*, 1992) were cloned but, as yet, there is no evidence for the existence of more than one cytosine DNA MeTase. The murine cDNA for DNA MeTase (cloned from erythroleukemia cells) was expressed in bacterial cells and shown to bear both maintenance and *de novo* methylation activities (Tollefsbo and Hutchison, 1995). Recent purification and expression in a baculovirus system of the murine DNA MeTase from erythroleukemia cells (Glickman and Reich, 1994; Xu *et al.*, 1995) demonstrated the presence of a single protein encoding the murine DNA MeTase in these cells, suggesting that previous reports of more than one form of

MeTase in murine erythroleukemia cells (Bestor and Ingram, 1983) could be explained as proteolytic digestion products of the 190 kDa protein (Xu *et al.*, 1995). On the other hand, homologous knockouts of the DNA MeTase resulted in dramatic, but not complete, inhibition of the DNA MeTase activity in homozygote embryos supporting the presence of other DNA MeTases (Li *et al.*, 1992). Cloning of the 5' genomic sequences of the DNA MeTase reveals some putative alternative splice sites, as well as alternative initiation sites that might encode different proteins (Rouleau *et al.*, 1992). However, no additional enzymes were described to date. Overexpression of the DNA MeTase cDNA results in hypermethylation, suggesting that the cDNA cloned by Bestor *et al.* (1988) could also induce *de novo* methylation patterns *ex vivo* (Wu *et al.*, 1993). Because the same enzyme is present in different cells showing different patterns of methylation, the critical question is how can one enzyme explain the large number of site- and tissue-specific methylation patterns?

5.3. The Semiconservative Model of Inheritance of Methylation Patterns: Maintenance vs. *de novo* Methylation

If different cells bear specific methylation patterns, there must be a mechanism responsible for replicating the pattern. This mechanism cannot be explained by differential MeTase activity, as described in Section 5.2. A simple model explaining maintenance of cell-specific patterns of methylation has been proposed (Razin and Riggs, 1980). Replication results in the generation of a hemimethylated double-stranded DNA, composed of a methylated parental strand and nonmethylated nascent strand. This hemimethylated DNA will now serve as a substrate for the DNA MeTase (Fig. 5). Razin and Riggs' model proposes that DNA methylation patterns are inherited during replication because the DNA MeTase is very efficient in methylating hemimethylated CpG moieties (maintenance methylation), but relatively inefficient in methylating nonmethylated substrates (*de novo* methylation). Therefore, only sites that are methylated in the parental strand will be methylated in the daughter strand (maintenance methylation), and sites that are not methylated in the parental strands will remain nonmethylated (inefficient *de novo* methylation). Hence, methylation patterns will be faithfully maintained. According to this model, the memory of the methylation pattern is carried by the methylation pattern of the template parental strand *per se* ("semiconservative inheritance"). Therefore, a simple nonsite-selective DNA MeTase can explain the faithful inheritance of specific methylation patterns. This model first predicts that the DNA MeTase enzyme is highly specific to hemimethylated strands and is supported by *in vitro* experiments that compared the methylation activity of a partially purified mammalian DNA MeTase using hemimethylated vs. nonmethylated substrates (Gruenbaum *et al.*, 1982). The model's second prediction is that in a differentiated somatic cell, *de novo* methylation activity will be extremely low because such an activity inevitably will result in a disruption of the methylation pattern. Therefore, when

a methylation of a site is lost, it will not be recovered because methylation is dependent on the presence of a methylated template. Therefore, DNA methylation patterns in somatic cells are predetermined and stable. To support this hypothesis, Stein *et al.* introduced exogenous methylated DNA and nonmethylated DNA into mouse somatic L cells by DNA-mediated gene transfer and assayed the pattern of methylation following the passaging of the cells for many generations. Originally, nonmethylated sites remained nonmethylated, and originally methylated sites remained methylated, at least partially (Stein *et al.*, 1982a,b). These data supports the hypothesis that in somatic cells, DNA methylation of the daughter strand is directly determined by the parental methylation pattern. This model can also explain how, in parental imprinting, an allelic methylation pattern is carried over through multiple generations from fertilization to adulthood. The allele that is originally methylated in gametogenesis will maintain its methylation pattern through multiple replication cycles. This is because DNA MeTase efficiently methylates a hemimethylated site (generated by replication of a methylated site). The nonmethylated allele will not be methylated in development, because DNA MeTase is very inefficient in methylating sites that are nonmethylated on both strands. However, as discussed in Section 3.3, the pattern of methylation of parentally imprinted genes cannot be explained by this simple mechanism.

5.4. Signals Other Than Hemimethylation Must be Involved in Determining Methylation Patterns

The "semiconservative inheritance" model is very appealing because of its simplicity; however, a long line of evidence has established that the memory of DNA methylation patterns is restricted not only by the state of methylation of the parental strand (reviewed in Szyf, 1991). First, it is clear that methylation patterns are altered in development by demethylation and *de novo* methylation (Monk *et al.*, 1987; Shemer *et al.*, 1991; Kafri *et al.*, 1992; Brandeis *et al.*, 1993b; Kafri *et al.*, 1993; Razin and Kafri, 1994) (Fig. 5). Because these processes result in specific alterations in methylation patterns, factors other than the original pattern of methylation must determine methylation patterns. Razin and Riggs (1980) suggested that these processes are determined by other enzymatic activities (a *de novo* methylase), and they differentiate between the propagation of a methylated pattern, determined by the methylation state of the parental strand (maintenance methylation) and events occurring during development (*de novo* methylation). Recently, Laird and Jaenisch (1994) have suggested that mammalian cells bear another DNA MeTase activity that is specific for *de novo* methylation. Whether or not an additional activity of that specificity exists remains an open question. However, it is clear that some methylation events must be determined by elements other than DNA MeTase *per se*, or the state of hemimethylation. If one takes parental imprinting as an example, what protects the methylation pattern of an imprinted allele from loss or addition of methyl groups at the time of em-

bryogenesis when general demethylation or *de novo* methylation occurs? It is now clear, as discussed in Section 3.3, that parentally imprinted genes form their methylation patterns *de novo* late in development, and that these methylation patterns have to be dictated by signals other than hemimethylation. Moreover, Sapienza *et al.* (1989) have shown that the pattern of imprinting is dependent on the genetic background of the other parent, suggesting that *trans*-acting factors also determine methylation patterns. A gene (Imprintor-1) that can control the parental imprinting of the *Tme* locus in mouse and, possibly, its methylation has been identified (Forejt and Gregorova, 1992). The fact that genetic factors other than DNA MeTase itself can determine methylation patterns has been shown in humans, as well. Heritable allele-specific and tissue-specific methylation patterns have been identified in 10 human loci. These genes must contain allele-specific signals in the germ line different from hemimethylation because their pattern of methylation is formed *de novo* in differentiated tissues (Silva and White, 1988).

5.5. Partially Methylated Sites in Clonal Tissues Are Inconsistent with the "Semiconservative" Model of Inheritance of Methylation Patterns

The well-established existence of "partially methylated" sites in clonal cell populations is inconsistent with the hypothesis that methylation of the parental DNA strand *per se* determines the inheritance of methylation patterns (Yisraeli and Szyf, 1984; Turker *et al.*, 1989). Partial methylation in a clonal population implies a loss of methyl groups during replication. If maintenance methylation is solely responsible for the inheritance of methylation, then each loss of a methyl group during a replication event must be irreversible. If *de novo* methylation is not specifically targeted, it will result in a drift in the methylation pattern. Although this well-established phenomenon was originally dismissed as representing different populations of cells, even in a seemingly single lineage group of cells, it is now clear that clonal populations of cells maintain partially methylated sites for many generations (Turker *et al.*, 1989). Moreover, when a fragment bearing a partially methylated site is cloned, loses its methylation pattern, and is reintroduced into the cell, it will acquire the same partial methylation pattern (Turker *et al.*, 1989). This strongly suggests that the tendency of this site to be methylated is determined by portable signals in the sequence itself, and each round of methylation is a specific *de novo* methylation event. Partial methylation of a site, therefore, reflects its probability to become methylated at each round of replication. Hemimethylated sites are also shown to be maintained as such for several generations (Toth *et al.*, 1990). The persistence of hemimethylated sites in a proliferating cell is inconsistent with the semiconservative model of inheritance of methylation patterns.

A clear example of site- and cell-specific *de novo* methylation in somatic cells is the specific *de novo* methylation of an exogenous nonmethylated transfected 21-hydroxylase gene in the adrenocortical cell line Y1 (Szyf *et al.*, 1989,

1990a). Other sequences are not methylated in Y1 cells, and the 21-hydroxylase gene is not methylated in other cells. Because there is no obvious difference in DNA MeTase between different cells, and because *de novo* methylation in Y1 cells occurs only at specific sequences, it suggests that sequence- and cell-specific signals for methylation exist in Y1 cells.

Both Razin and Riggs's (1980) and Laird and Jaenisch's (1994) models differentiate between *de novo* and maintenance methylation as two fundamentally different modes of transmission of the methylation pattern. I previously suggested a unifying hypothesis to explain the generation of new methylation patterns by *de novo* methylation and demethylation, their propagation in specific lineages, and their paternal inheritance. According to this model, DNA methylation patterns are determined by an interaction between signals in the DNA sequence and proteins that interact with these signals and target-specific sites for DNA methylation or demethylation (Szyf, 1991).

5.6. DNA Methylation Patterns Are Determined by an Interaction Between Signals in the DNA "Centers of Methylation" and trans-acting Proteins that Interact with These Signals: A Model

The model that I have previously proposed suggests that DNA methylation, like transcription and other genomic functions such as DNA replication, is determined by DNA cis-acting signals ("centers of methylation") and trans-acting factors that interact with these signals and target juxtaposed sequences for methylation. What is the identity of these factors? Perhaps they are members of the ubiquitous histone protein family such as H1 (Higurashi and Cole, 1991; Johnson *et al.*, 1995) or nonhistone proteins such as high mobility groups (Paranjape *et al.*, 1994). Although some data exist relating to the question of whether or not the binding of histones can alter methylation patterns and the efficiency of the methylation reaction (Caiafa *et al.*, 1991; D'Erme *et al.*, 1993; Santoro *et al.*, 1995), it is still unclear as to whether or not inactive chromatin structure by itself can alter accessibility to DNA MeTase. Other "modifying proteins," which are cell- and sequence-specific, also exist (Szyf *et al.*, 1989). The mode of action of these putative cell-specific proteins, in turn, might involve more general proteins, such as histone-like proteins mentioned in Section 4.1, as downstream effectors. For example, a cell-specific repressor can cause the precipitation of an inactive chromatin structure, and this, in turn, might directly enhance DNA methylation by a general mechanism. A number of studies by Turker's group have demonstrated a cis-acting "*de novo* methylation center" in the upstream region of the mouse adenosine phosphoribosyl transferase (*aprt*) gene (Turker *et al.*, 1991; Mummaneni *et al.*, 1993). This sequence can also lead to an inactivation of the *aprt* gene, supporting the hypothesis that centers of methylation can also function as centers of inactivation (Mummaneni *et al.*, 1995). The transfected *aprt* gene-methylation pattern mimics the pattern of methylation of

endogenous sites, demonstrating clearly that the memory of the methylation pattern is determined by the sequence itself and the repertoire of factors that are present in the cell, and interact with it. Additional cis-acting methylation enhancing sequences were identified in exon 7 of the *p53* gene (Magewu and Jones, 1994) and the α -fetoprotein control region (Hasse *et al.*, 1992; Hasse and Schulz, 1994). In addition to signals that enhance *de novo* methylation, other cis-acting signals that protect juxtaposed sequences from *de novo* methylation, "hypomethylation signals," were identified in the CpG island of the *thy-1* gene (Szyf *et al.*, 1990b) and *hprt* (Brandeis *et al.*, 1994). Recent experiments have identified a binding site for the transcription factor Sp-1 as critical for the activity of the cis-acting hypomethylation signal contained in the *hprt* 5' region (Brandeis *et al.*, 1994; Macleod *et al.*, 1994). One possible explanation is that the binding of a transcription factor precipitates a chromatin structure that alters the accessibility to DNA MeTase (Szyf, 1991). A protein from human sperm nuclei that specifically binds to Alu DNA repeats and selectively protects Alu sequences from methylation *in vitro* has been purified recently (Chesnokov and Schmid, 1995). This protein might be the first representative of a class of proteins involved in determining *de novo* DNA methylation patterns.

Other models propose that *de novo* methylation and maintenance methylation are independent and qualitatively different events (Razin and Riggs, 1980; Laird and Jaenisch, 1994); my model suggests that there is no need to differentiate between the two processes. It is clear that the DNA MeTase exhibits *in vitro* higher affinity towards hemimethylated DNA (Gruenbaum *et al.*, 1982); it is also clear that it can carry out both *de novo* and maintenance activities (Gruenbaum *et al.*, 1982; Bestor and Ingram, 1983). The specificity of both processes is proposed to be determined by interaction of cis- and trans-acting factors. A change in the cellular repertoire of these factors can result in changes in methylation. Thus, it is proposed that methylation is a dynamic, rather than a static, process even in somatic cells and is not rigidly fixed by the legacy of the methylation pattern.

The model proposed here suggests a circular relationship between DNA methylation and different genome functions, such as gene expression: protein binding determines DNA methylation patterns and that, in turn, alters DNA-protein interactions. This model can also explain why, in certain instances, changes in methylation precede alterations in gene expression whereas, in other cases, changes in methylation follow gene expression changes, as discussed in Section 3.2.1 (Fig. 4) (see, for an example, Benvenisty *et al.*, 1985).

I, therefore, suggest a different approach to the question of whether DNA methylation is a result or consequence of changes in gene expression. It is clear that DNA methylation patterns can be established independent of gene expression (Szyf *et al.*, 1989; Brandeis *et al.*, 1994). However, because both DNA methylation and gene expression are determined by DNA-protein interactions, these processes can converge at different points. In the previous section,

I have suggested that the answer to the question of whether methylation is a cause or effect of gene expression could be answered only after we understand how DNA methylation patterns are generated. If DNA methylation patterns are determined by a mechanism different from gene expression, they could not be a consequence of gene expression. It is clear that one of the most critical questions in DNA methylation is identifying the proteins involved in fashioning the methylation pattern. These proteins most probably will serve as important targets for therapeutics aimed at specifically modifying methylation at a site or a subset of sites.

What makes DNA methylation an attractive candidate to transmit epigenetic information is that it is a stable covalent modification of DNA, and that it could be faithfully inherited by means of the maintenance DNA MeTase (Razin and Riggs, 1980). However, if, as the model proposed here suggests, DNA methylation is determined by proteins, what information does DNA methylation add that the proteins that create the methylation pattern cannot convey? One proposal is that methylation amplifies a signal initiated by a change in DNA-protein interactions. Interaction of a "modifying" protein with a center of methylation will trigger methylation. Next, methylation will induce a change in chromatin structure that, in turn, will enhance spreading methylation beyond the initial signal. This, in turn, will result in spreading the signal across wide areas of the genome by carrying it through the germ line, as is done in parental imprinting, and by maintaining a long-term memory after loss of the initial signal (Szyf, 1991).

5.7. The Control of the Level of DNA Methyltransferase Activity as a Site for Regulation of DNA Methylation Patterns

The model suggested in Section 5.6 can explain the generation and inheritance of site-specific DNA methylation patterns. The traditional approach to the study of DNA methylation patterns was to focus on specific sites and correlate their state of methylation with patterns of gene expression (Razin and Riggs, 1980; Yisraeli and Szyf, 1984; Cedar, 1988). It is now clear, however, that genome-wide changes in methylation occur during early development; a genome-wide demethylation at the morula stage is followed by genome-wide hypermethylation at the pregastrula stage (Kafri *et al.*, 1993; Razin and Kafri, 1994). Similarly, genome-wide changes in methylation occur in differentiating F9 teratocarcinoma cells (Bestor *et al.*, 1984; Razin *et al.*, 1984), erythroleukemia cells (Razin *et al.*, 1986), and Burkitt lymphoma cells (Szyf *et al.*, 1985). Of most relevance to our discussion is the widespread hypomethylation (Gama-Sosa *et al.*, 1983b; Feinberg and Vogelstein, 1983; Feinberg *et al.*, 1988) and hypermethylation observed in cancer cells (Baylin *et al.*, 1986, 1991; Nelkin *et al.*, 1991; Makos *et al.*, 1992). Can site-specific factors explain the genome-wide changes in methylation observed in cancer cells and differentiating cell lines? An obvious candidate to play a role in controlling the general state of DNA methylation is the level of DNA MeTase activ-

ity in the cell (Szyf *et al.*, 1984). To play such a role, DNA MeTase activity should be limiting and regulated by central cellular control points. If this is true, it raises the possibility that changes in the level of DNA MeTase activity occur at different points during development and could be altered in certain disease states, such as cancer. Understanding the mechanisms regulating DNA MeTase activity might provide us with clues regarding the biological roles of DNA methylation. This possibility is attractive from the pharmacological point of view because modulating the activity of an enzyme should be more feasible pharmacologically than regulating the activity of hitherto unidentified *cis*-acting factors.

5.8. Regulation of DNA Methyltransferase Gene Expression

Why are sites partially methylated? The fact that they are methylated in a fraction of a clonal population of cells implies that they are potential substrates for DNA methylation. One simple explanation is that the activity of DNA MeTase is limiting and, therefore, some sites escape methylation at every round of methylation.

One interesting model for studying the role of limiting DNA MeTase activity in a cell is the Dam methylase in *E. coli*. In *E. coli*, both Dam and Dcm methylases are limiting (Szyf *et al.*, 1984), and maintaining a limiting level of MeTase activity is critical for keeping the origin of replication in a hemimethylated state through replication (Boye and Lobner-Olesen, 1990; Campbell and Kleckner, 1990). The hypothesis that the level of DNA MeTase is limiting in mammalian cells is supported by the observation that a small elevation of cellular DNA MeTase levels by forced expression of an exogenously introduced DNA MeTase into NIH 3T3 cells results in a significant change in the methylation pattern (Wu *et al.*, 1993). Additionally, limited inhibition of DNA MeTase by expression of an antisense RNA to the DNA MeTase mRNA results in a reduction in the level of methylation in the genome (Szyf *et al.*, 1992; MacLeod and Szyf, 1995). However, it is clear that there is no simple correlation between the level of DNA MeTase in the cell and the pattern of methylation of DNA (Kautiainen and Jones, 1986; Bestor *et al.*, 1988; Carlson *et al.*, 1992). This is not surprising because, as discussed in Section 5.6, it is clear that *cis*-acting signals, as well as *trans*-acting modifiers, are involved in the fashioning of DNA methylation patterns. Therefore, it has been suggested that DNA methylation patterns are determined by an interplay between the level of DNA MeTase activity and site-specific signals (Szyf *et al.*, 1984; Szyf, 1991). A new study by Doerfler's group suggests that the sequence is only one component of many factors that are involved in initiating *de novo* methylation in an integrated adenovirus sequence (Orend *et al.*, 1995).

If the level of DNA MeTase activity can determine DNA methylation patterns, DNA MeTase gene expression should be regulated. There should also be signals for altering the level of DNA MeTase activity when a programmed change in DNA methylation occurs. Several reports have established

that the DNA MeTase activity is regulated with the cell cycle, and is mainly expressed in growing cells and induced at the G1-S boundary (Szyf *et al.*, 1985; Singer-Sam *et al.*, 1990b; Szyf *et al.*, 1991; el-Deiry *et al.*, 1991). The nuclear localization of the DNA MeTase is similarly controlled with the S phase of the cycle (Leonhardt *et al.*, 1992). Other reports have demonstrated regulation of DNA MeTase levels at early developmental stages of both vertebrates and sea urchin (Tosi *et al.*, 1995), such as the high activity reported in mouse oocytes and zygotes (Monk *et al.*, 1991; Carlson *et al.*, 1992). However, there is a progressive loss of activity between 8-cell stage embryos and blastocysts (Monk *et al.*, 1991; Croteau and Menezo, 1994). Although the progression of embryos towards the blastula stage is accompanied with genome-wide demethylation, the changes in DNA MeTase activity do not directly correlate with the state of genomic DNA methylation (Carlson *et al.*, 1992), which is in accordance with the hypothesis presented in Section 5.6 that additional *cis*- and *trans*-acting factors are involved. An interesting example of regulated changes in the level of DNA MeTase mRNA and activity during development is its regulation during spermatogenesis (Trasler *et al.*, 1992; Benoit and Trasler, 1994).

5.9. Regulation of DNA Methyltransferase by Oncogenic Signaling Pathways; Hypermethylation is a Downstream Component of an Oncogenic Program

It is clear that DNA MeTase gene expression is regulated at a number of levels to respond to different classes of signals, maintaining limiting levels of expression under normal conditions, and responding to needs to increase DNA methylation under other situations. The cell cycle regulation of the DNA MeTase is controlled mainly at the posttranscriptional level (Szyf *et al.*, 1991). An analysis of the promoter region of the DNA MeTase gene has provided us with interesting insights, not only into the regulatory events controlling MeTase gene expression, but also the possibility of cross talk between DNA methylation and other cellular signaling pathways (Rouleau *et al.*, 1992, 1995). The 5' upstream regulatory region of the DNA MeTase is composed of a minimal promoter that bears no TATA, CG-rich Sp-1 elements or the previously characterized initiator, a GT-repeat with potential repressor functions and a cluster of AP-1 recognition sequences (Rouleau *et al.*, 1992). A 100 bp sequence bearing three AP-1 recognition sequences (at -1744 to -1650) is responsible for the induction of DNA MeTase promoter activity (Rouleau *et al.*, 1995). AP-1 is a transactivation complex composed of a heterodimer of the Fos and Jun DNA-binding proteins or a homodimer of Jun, which is activated by the Ras oncogenic signaling pathway (Angel and Karin, 1991; Binetruy *et al.*, 1991; Lowy and Willumsen, 1993). The regulation of the DNA MeTase by a major oncogenic signaling pathway provided the first molecular link between oncogenic signaling pathways and mechanisms regulating DNA MeTase activity, and positioned the DNA MeTase as a downstream

effector of oncogenic pathways. Using the Y1 adrenocortical cell line as a model system to study the biological consequences of the regulation of DNA MeTase by the Ras signaling pathway, MacLeod *et al.* have shown that a down-regulation of the Ras-AP-1 pathway leads to the inhibition of expression of DNA MeTase mRNA, decreased DNA MeTase activity, DNA hypomethylation, as well as inhibition of cellular transformation. Overexpression of oncogenic Ras leads to induction of DNA MeTase activity and DNA methylation (MacLeod *et al.*, 1995). The hyperactivation of DNA MeTase by Ras is critical for maintaining the transformed state of Y1 cells, because inhibiting the level of DNA MeTase activity in the cell by expression of an antisense to the DNA MeTase or by 5-azaC treatment results in the inhibition of DNA methylation and reversal of the transformed state (MacLeod and Szyf, 1995). The study of the mechanisms regulating DNA methylation patterns points to a possible biological role of DNA methylation as downstream effector of the Ras oncogenic signaling pathway (Szyf, 1994).

One interesting example of a physiological mechanism induced by the Ras signaling pathway is the cellular response to the activation of tyrosine kinase receptors, such as the nerve growth factor receptor (Noda *et al.*, 1985). Induction of DNA MeTase could be a result of activation of this pathway during development, but might also play a part in the cellular response of mature cells to ligands for these receptors. These results raise the interesting possibility that some receptor activation could induce changes in the covalent modification of the genome. This might be a mechanism for long-term effects induced by receptors that trigger the Ras signaling pathway. DNA MeTase might be regulated by signaling pathways at the posttranscriptional level. Dibutyl cyclic AMP was found to suppress DNA MeTase in the human monoblast cell line U397, and phorbol ester 12-O-tetradecanoyl phorbol-13-acetate was shown to stimulate DNA MeTase (Soultanas *et al.*, 1993). The possibility that DNA MeTase activity and DNA methylation pattern could be altered by extracellular and intracellular signals raises the possibility that methylation patterns exhibit more plasticity in mature somatic tissues than previously thought. If this is true, we should revise our understanding of the biological role of methylation patterns from the static maintainers of epigenetic information to dynamic regulators of the genome that are constantly influenced by extracellular and cellular signals.

Recent data showing that stabilization of DNA MeTase levels and CpG island hypermethylation precedes Simian virus 40-induced immortalization of human fibroblasts, suggests that other growth-signaling pathways, such as T antigen, might control the level of expression of the DNA MeTase (Vertino *et al.*, 1994). T antigen appears to inhibit the function of the Rb tumor-suppressor protein, which is a critical regulator of cell cycle progression (Riley *et al.*, 1994). It is tempting to speculate that tumor suppressors such as the Rb protein play an inhibitory role in controlling DNA MeTase activity.

5.10. The Role of DNA Demethylation

It is clear that the DNA MeTase is not the only enzyme involved in generating DNA methylation patterns. The methylation pattern is established by sequential demethylation at the preimplantation stage and waves of *de novo* methylation and site-specific demethylation events (Monk *et al.*, 1987; Brandeis *et al.*, 1993a; Kafri *et al.*, 1993; Chapman *et al.*, 1984). The biochemical mechanisms that are responsible for demethylation are still unclear. Demethylation could come about by a passive process, i.e., inhibition of methylation during the process of DNA replication, as has been suggested by the Razin and Riggs model (Razin and Riggs, 1980). However, a series of observations have demonstrated that an active process of demethylation that does not involve replication occurs in mammalian cells. Wilks *et al.* (1984) demonstrated active demethylation of the *vitellogenin II* promoter in response to estrogen induction in hormone-responsive tissues in the chicken. Epstein-Barr virus genomes undergo demethylation upon induction of a lytic cycle in Epstein-Barr virus producer lines, before initiation of replication (Szyf *et al.*, 1985). Using transient transfection assays, it has been shown that exogenous α -actin gene is actively demethylated at specific sites in C2C12 cells (Paroush *et al.*, 1990) and that CpG islands are specifically actively demethylated in mouse embryonal cell lines (Frank *et al.*, 1991). Similar to *de novo* methylation (Szyf *et al.*, 1989, 1990b), demethylation is directed by specific signals in the DNA sequence (Paroush *et al.*, 1990; Lichtenstein *et al.*, 1994). Recent elegant studies followed the fate of *in vitro* methylated sequences introduced into mouse zygotes during development, and demonstrated that the sequences were actively demethylated at the preimplantation stage (Kafri *et al.*, 1992, 1993). In addition to site-specific demethylation, there is evidence that genome-wide demethylation occurs during cellular differentiation (Razin *et al.*, 1984, 1985; Szyf *et al.*, 1985; Razin *et al.*, 1986). The fact that demethylation could be genome-wide raises the possibility that, similar to methylation, demethylation is controlled by the availability of a general demethylase activity (Szyf, 1994; Szyf *et al.*, 1995).

What is the biochemical mechanism behind this well-established biological phenomenon? Surprisingly, the answer to this question has remained elusive. No activity that *bona fide* demethylates DNA by removing methyl groups from methylated cytosines has been identified to date. Gjerset and Martin reported in 1982 the presence of a DNA-demethylating activity in murine erythroleukemia cell nuclei. This work measured the release of methylated moieties from *in vitro* methylated DNA, but it is not clear whether this activity removed methyl group *per se* or excised the methylated base. Moreover, the paper did not establish whether or not this activity was specific to methylated cytosines (Gjerset and Martin, 1982). This work has not been followed up. More recently, Razin *et al.* (1986) have suggested that demethylation in Friend erythroleukemia cells during differentiation can occur by the removal of the methylated cytosine base and its replacement with nonmethylated cytosines: "excision and replacement." A glycosylase activity that can remove

5-methylcytosine bases from DNA has been identified in nuclear extracts from Hela cells (Vairapandi and Duker, 1993). However, it is not clear whether or not this activity is specific to methylated cytosines rather than pyrimidines. Another possible indirect mechanism for demethylation is a 5-methylcytosine-directed endonuclease activity in chicken embryos and murine 10 T1/2 cells (Jost, 1993; Jost and Jost, 1994), which can trigger the excision and replacement of methylated cytosines in hemimethylated DNA in the presence of deoxy nucleotide triphosphates and a DNA polymerase. When this activity has been purified, Jost *et al.* (1995) discovered that this enzymatic activity is a combination of 5-methylcytosine glycosylase and apyrimidine-endonuclease.

Perhaps this combination of glycosylase-AP endonuclease is the long-sought 5-methylcytosine demethylase. However, it is still possible that another activity is responsible for demethylation. One indication that the activity identified by Jost is a "repair" demethylation activity, rather than a demethylase responsible for the removal of "methyl" groups from both strands of DNA, is its preference for a hemimethylated substrate. Jost suggested that this activity, like other "mismatch repair" activities, is required for removal of methyl groups that are aberrantly introduced into the nascent strand of DNA during replication (Jost *et al.*, 1995). Future experiments will have to address this point.

Whatever the biochemical mechanism of demethylation, the important biological question is how this activity is involved in the generation and maintenance of methylation patterns. Because extensive demethylation is known to occur in early development (Razin *et al.*, 1984; Monk *et al.*, 1987; Kafri *et al.*, 1993), we chose the mouse embryonal carcinoma cell line P19 as a model (Szyf *et al.*, 1995). Using transient transfection assays, it has been shown that an *in vitro* methylated SK-plasmid bearing no sequence homology to any eukaryotic genes becomes fully demethylated between 1 and 2 days after transfection into P19 cells. The demethylating activity is not strictly sequence-selective and is independent of DNA replication. Even though the biochemical mechanism involved in removal of methyl groups is still unclear, these results demonstrate that early embryonic cells possess an activity that can explain the active demethylation occurring in early development. Two questions remain. First, what determines the developmental stage specificity of demethylation (Kafri *et al.*, 1992)? Second, what determines the site specificity of demethylation? I previously have suggested that, similar to methylation, demethylation can be controlled by the availability of the demethylase and by *cis*-acting signals and *trans*-acting factors that gate the accessibility of the demethylase to methylated sequences (Szyf, 1991, 1994). *Cis*-acting sequences that can modulate site-specific demethylation have been identified (Paroush *et al.*, 1990; Lichtenstein *et al.*, 1994). Recent data also supports the hypothesis that the availability of the demethylase is regulated by central cellular-signaling pathways. Expression of oncogenic Ras in P19 cells induces a dramatic induction (10- to 100-fold) of demethylation activity, resulting in genome-wide demethylation (Szyf *et al.*, 1995).

The identification of a general demethylation activity and its regulation by Ras can help solve the paradox that, in many cancer cells and early in development, DNA MeTase is hyperactivated and genome-wide hypomethylation is also observed. If both DNA MeTase and demethylase are induced by oncogenic signaling pathways, demethylation of specific subsets of sites and hypermethylation of others could co-exist if the two enzymes show different substrate specificities (Szyf, 1994).

One possible example of two distinct subsets of methylation sites that show opposite propensities to be methylated are the CpG-rich islands (Bird *et al.*, 1985) (regions of DNA usually at the 5' of housekeeping genes that are nonmethylated in most tissues) vs. CpG sites positioned around tissue-specific genes (Yisraeli and Szyf, 1984; Razin and Kafri, 1994). In early embryonic stem cells, CpG islands are demethylated (Frank *et al.*, 1991) and protected from *de novo* methylation (Szyf *et al.*, 1990b). However, other sites are *de novo* methylated. In cancer cells, on the other hand, CpG islands are *de novo* methylated (Baylin *et al.*, 1986; Jones *et al.*, 1990b; Rideout *et al.*, 1994) and other sites are hypomethylated (Feinberg and Vogelstein, 1983). Two alternative hypotheses can explain this switch in the methylation state of these classes of CpG sites. One hypothesis is that different MeTases or demethylases are induced in early embryogenesis, as opposed to cancer. Alternatively, the switch in methylation specificity is determined by a change in *trans*-acting factors interacting with *cis*-acting signals that determine the accessibility of these sites to the methylation or demethylation machinery. One such example is the *cis*-acting signal located in the CpG island of the *thy-1* promoter and *hprt* gene (Szyf *et al.*, 1990b; Brandeis *et al.*, 1994) that can protect CpG sites from *de novo* methylation in early embryogenesis. Differential expression of the factor(s) interacting with these sequences can explain the differences in CpG island methylation observed in cancer cells vs. early embryonic cells. Regulated induction of demethylase activity at different points in development and in carcinogenesis can explain the genome-wide demethylation observed at these stages. Local signals and proteins interacting with them could also explain the discrete site-specific demethylation of tissue-specific genes during development and carcinogenesis. Identifying the molecular mechanism regulating DNA demethylation activity and identifying demethylation signals and factors that interact with them is one of the most important challenges of the DNA methylation field.

5.11. Cancer and Methylation. The Level of DNA Methyltransferase and Demethylase Activities as Control Points of Oncogenic Programs

Is the aberrant methylation observed in cancer cells a critical component of an oncogenic program, or just a reflection of the chaotic state of a cancer cell? Whereas many studies have pointed out to aberrant methylation patterns in cancer, as discussed in Sections 3.4.2 and 3.4.3, the prevailing under-

standing is that these changes reflect the general deterioration of the cell rather than a programmed event. Some might argue that methylation of tumor-suppressor genes or CpG islands (Baylin *et al.*, 1986; Jones *et al.*, 1990b; Rideout *et al.*, 1994) confers some selective advantage, and is selected for by the oncogenic process. Another possible model, consistent with random events that confer selective advantage, is that hypermethylation enhances the probability of conversion of methylated cytosines to thymidine (Jones *et al.*, 1992). Recently, the possibility that DNA MeTase itself might bear a deamination activity (Shen *et al.*, 1992) has been suggested to link the high DNA MeTase activities observed in cancer and mutagenesis (Laird and Jaenisch, 1994). The implication of these models is that relatively little could be done pharmacologically to reverse methylation-induced carcinogenesis. If methylation leads to stable mutations, which are a critical component of the cancer process, then the process is irreversible. The study of the regulation of DNA methylation patterns discussed in this section led to the suggestion that activation of DNA MeTase activity and demethylase activities is a critical component of the oncogenic program (Szyf, 1994), rather than a random, but selected, process. If DNA methylation patterns are programmed by regulation of DNA MeTase and demethylase activities, there is the possibility of reversing the oncogenic program by inhibiting the excess DNA MeTase and demethylase activities. The pharmacological implications of this hypothesis are obvious. The fact that one could reverse the transformed state of a cell by inhibiting DNA methylation (MacLeod and Szyf, 1995) supports this hypothesis, and the potential therapeutic role of DNA MeTase inhibitors. The level of DNA MeTase activity, therefore, might be a nodal control point over the oncogenic program. Thus, the study of the elements regulating DNA methylation patterns points out to a potential new anticancer pharmacological target.

5.12. DNA Methylation as a Control Point Over DNA Replication: A Model

How can an increase in DNA methylation play a causal role in an oncogenic program? One possibility is that an increase in DNA methylation activity leads toward a programmed methylation of a large number of tumor-suppressor loci, and an inhibition of methylation reverses this process (Szyf, 1994). Hyperactivation of DNA MeTase might also be involved in the methylation and permanent inactivation of cell-specific genes that are not necessary, or might even slow down cellular growth, e.g., the 21-hydroxylase gene in adrenocortical tumors (Szyf *et al.*, 1990a; Mellon *et al.*, 1994), or the suppression of prolactin gene expression in pituitary tumor cell lines (Arnold *et al.*, 1991). Alternatively, the fact that DNA MeTase activity is regulated with the cell cycle (Szyf *et al.*, 1991), and that it is regulated by cellular signaling pathways that are hyperactivated in oncogenesis, raises the question of whether or not DNA methylation is directly involved in control of the cell cycle. The results by Tasheva and Roufa (1994), showing hypermethylation of an origin of replication

in growing cells and hypomethylation of the same sequences in arrested cells, point to the interesting possibility that DNA methylation can directly control replication. This might suggest a direct role for hyperactivation of DNA MeTase in the control of growth of cancer cells. I would like to suggest the following model: Replication is initiated from methylated origins (Tasheva and Roufa, 1994). In cell cycle-arrested cells, DNA MeTase activity is low, as we have shown before (Szyf *et al.*, 1991) and, therefore, the origins of replication are hypomethylated (Tasheva and Roufa, 1994). Induction of DNA MeTase activity at the G1-S boundary (Szyf *et al.*, 1991) and methylation of origins of replication are required for initiation of replication. After replication is initiated, the origin becomes hemimethylated (the nascent strand is not methylated). The origin remains inaccessible to the DNA MeTase throughout S, probably by its association to the putative fixed "replication factories" (Coverly and Laskey, 1994), as is the case in *E. coli* (Campbell and Kleckner, 1990). After replication is completed, the origin is methylated and becomes a substrate for a new round of DNA replication. Tasheva and Roufa (1994) suggested that, when a cell retreats from the cell cycle, a demethylase removes the methyl groups from the origin. Aberrant induction of the DNA MeTase by activation of the Ras signaling pathway will result in methylation of origins of replication and initiation of DNA replication. Overexpression of DNA MeTase in cancer cells results in premature methylation of origins, causing firing of normally silent origins, also explaining the chromosomal abnormalities observed in cancer cells.

5.13. Summary: Methylation Patterns Reflect an Equilibrium Between Methylation and Demethylation and Site-Specific Signals

Based on the previously published observations and hypotheses discussed in this section, I will like to suggest the following model: The final methylation pattern is an equilibrium between methylation and demethylation. These two reactions express reverse substrate specificity. The final pattern of methylation will reflect the balance between the availability of both enzymes and the local changes in chromatin structure. Both methylation and demethylation machineries are regulated by cellular control pathways, and their expression is coordinated with the state of growth and development. Many questions remain. First, is there a specific enzyme responsible for *de novo* methylation in addition to the maintenance MeTase, or are both activities carried out by the same enzyme? The model suggested above does not require the presence of two distinct enzymatic activities. However, the possibility that other enzymatic activities exist cannot be excluded at this stage (Laird and Jaenisch, 1994). An additional question is whether or not the nonCpG methylation (Tasheva and Roufa, 1994; Clark *et al.*, 1995) observed in mammalian cells is also performed by the same MeTase? The nature of the *cis*-acting methylation and demethylation signals and centers, as well as the factors interacting with

these signals, is still vague and most probably will be better characterized in the near future.

6. THE PHARMACOLOGICAL POTENTIAL OF INHIBITORS OF DNA METHYLATION

6.1. Genetic and Parentally Imprinted Diseases

The discussion of the possible mechanisms by which methylation patterns are regulated provides us with some basic concepts regarding the therapeutic potential of modulating methylation patterns and possible strategies for drug intervention. DNA methylation stabilizes the repression of genes and exogenously introduced genetic material, as discussed in Section 4. It is possible that some congenital deficiencies in gene expression result from aberrant methylation rather than genetic alterations, e.g., Rb (Ohtani-Fujita *et al.*, 1993) and WT (Royer-Pokora and Schneider, 1992; Taniguchi *et al.*, 1995). These cancers are excellent candidates for demethylation therapy. The involvement of *IgfII*, a parentally imprinted gene, in some WTs provides us with another paradigm on how methylation could be involved in genetic diseases. Parentally imprinted genes are different from most of our genome because only one allele is expressed. Mutation of the active allele will result in complete loss of function, even though the other allele is genetically intact, but repressed by methylation. In some parentally imprinted genetic diseases, demethylation could activate the parentally silenced allele. Demethylating agents are of potential therapeutic use in this family of genetic diseases (Fig. 6). Another class of genetically-inherited diseases is thalassemia and sickle cell anaemia. In sickle cell anaemia, the adult mature β -globin protein is rendered defective by a point mutation, resulting in distorted red blood cells (Ingram, 1956). In thalassemia, there is a deficient synthesis of β -globin, caused by mutations in the β -globin gene (Orkin *et al.*, 1982). Mammals bear a set of fetal globin genes that are developmentally switched off; methylation is possibly one mechanism of stabilizing the repressed state (Shen and Maniatis, 1980; Busslinger *et al.*, 1983; Mavilio *et al.*, 1983; Enver *et al.*, 1988). Demethylation could be used to reactivate the fetal γ -globin, which is nonmutated in these patients. Reactivation of fetal globin genes will result in the generation of a population of red blood cells that express nonmutated haemoglobin chains (Fig. 7). 5-AzaC and 5-azadC were used successfully in the past to activate the fetal globin genes in primates (DeSimone *et al.*, 1982) and sickle cell anaemia patients (Ley *et al.*, 1982). These clinical trials were discontinued because of other side effects of 5-azadC, as discussed in Section 6.3. Recently, hydroxyurea (an inhibitor of ribonucleosidediphosphate reductase) has been used effectively to treat sickle cell anaemia, suggesting the activation of fetal globin by a different mechanism: possibly cytotoxicity results in reprogramming red blood cell maturation (Platt *et al.*, 1987). Because hydroxyurea is a cytotoxic agent with a wide range of adverse effects, it will be of advantage to have at hand agents that can specifically inhibit DNA methylation with limited cytotoxicity.

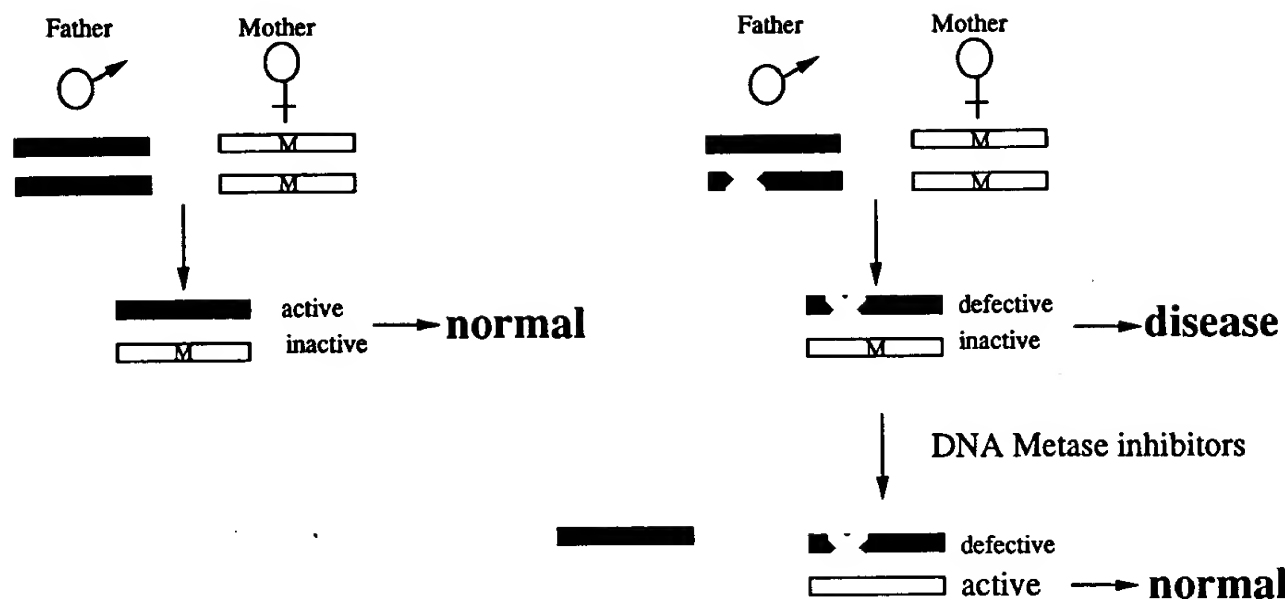


FIGURE 6. Activation of parentally imprinted genes by DNA MeTase inhibitors. Parentally imprinted genes are expressed when inherited from one parent (in this model, the father). Under normal conditions, the allele inherited from the other parent (the mother) is repressed by a mechanism involving methylation (M). If the person inherited a germ line mutation from one parent (father) and the other allele is parentally inactivated by methylation, there is a complete loss of function of this gene. Treatment with DNA MeTase inhibitors will result in activation of the parentally repressed allele and restoration of function.

6.2. Gene Therapy

It has been observed, as discussed in Section 3.5, that exogenously introduced genes are inactivated *in vivo*, and it has been suggested that this inactivation involves *de novo* methylation. Doerfler (1991) has suggested that *de novo* methylation might be an immune response at the cellular level, aimed at protecting cells from the introduction of foreign genetic material. Adenoviruses (Sutter and Doerfler, 1980) and retroviruses (Challita and Kohn, 1994) have been shown to undergo *de novo* methylation *in vivo*. Although it is not clear whether or not *de novo* methylation is the primary inactivating event (Harbers *et al.*, 1981; Gautsch and Wilson, 1983), it is possible that *de novo* methylation stabilizes the repressed state. The repression of retroviral vectors is especially frustrating when retroviral vectors are used for *in vivo* delivery of genes (Challita and Kohn, 1994). Inhibition of retroviral 5' MoMuLV-LTR-driven exogenous genes was observed in primary fibroblast (Palmer *et al.*, 1991) and hematopoietic cells (Williams *et al.*, 1986; Kaleko *et al.*, 1990). Two different strategies could be utilized to overcome this problem. First, *cis* signals, which protect adjacent sequences from methylation (Szyf *et al.*, 1990a), could be engineered into the delivery vectors, in addition to a deletion of negative control regions (Flanagan *et al.*, 1989; Challita *et al.*, 1995). Such an approach recently has been shown to increase the expression and decrease DNA methylation of an MoMuLV-LTR-driven neomycin resistance gene in F9 embryonic carcinoma cells (Challita *et al.*, 1995). Multiple *cis*-acting sequences determine the state of methylation and expression of retroviral vectors in accordance with the hypothesis that both positive ("centers of methylation") and negative ("centers of hypomethylation") *cis*-acting sequences determine methylation

patterns (Szyf, 1991). The emerging significance of these sequences, in the repression of exogenous gene expression, underscores the importance of identifying and characterizing these sequences and proteins interacting with them. Possible future developments of gene therapy might involve

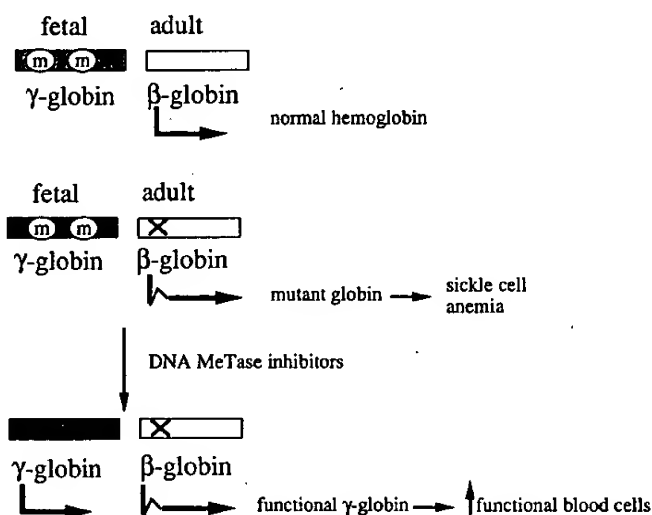


FIGURE 7. Activation of clinically relevant targets by DNA MeTase inhibitors. In sickle cell anaemia and thalassemia, the mature β -globin contains mutations, but the fetal γ -globin is intact. The expression of the γ -globin gene is developmentally repressed by a mechanism that involves DNA methylation. As the patients' hematopoietic cells bear the transcription factors required to express both the adult and fetal globin genes, demethylation should result in activation of the fetal globin and expression of functional globin. Methylatable sites are indicated by an open circle, methylation by M, mutation by an X, and transcription by an horizontal arrow.

generating modified vectors that had deleted the *de novo* methylation signals, and bear a number of *cis*-hypomethylating signals. In the long term, inhibitors of specific *trans*-acting factors inducing or repressing methylation of specific sites might also be developed. The second pharmacological approach is to use inhibitors of DNA MeTase to partially inhibit methylation and activate the repressed genes. The disadvantage of this approach is the lack of specificity and the fact that a general demethylation will be induced systemically. The advantage of the approach is its simplicity. The whole question of the potential risk involved in general demethylation therapy will be discussed below.

6.3. Cancer: Hypomethylation vs. Hypermethylation

Whereas there is no question that methylation patterns are changed in cancer cells, the true meaning of these changes has baffled the field for the last two decades. Because the acceptable dogma is that methylation induces gene repression and cancer involves activation of oncogenes, it is logical to propose that hypomethylation is involved in carcinogenesis (Feinberg and Vogelstein, 1983; Counts and Goodman, 1994). Whereas the original "site-specific" focus (Razin and Riggs, 1980) leads to the search for specific hypomethylation of oncogenic sequences, the surprising observation is that hypomethylation is global and not limited to oncogenes *per se* (Feinberg and Vogelstein, 1983; Feinberg *et al.*, 1988). The pharmacological implication of this model is that demethylating agents, such as 5-azaC or 5-azadC, are potentially carcinogenic and, therefore, should not be used in therapy. The most serious consequence of this hypothesis is the termination of clinical trials with 5-azaC in sickle cell anaemia. As recently as 1995, it has been suggested that 5-azadC should not be used in sickle cell anaemia because of its potential carcinogenicity (Platt, 1995). Because the mechanism responsible for hypomethylation in cancer is unknown and because most of the field is focused on correlative studies, the pharmacological potential of DNA methylation modifiers has been left untouched for more than two decades. The main activity in the field has been to provide direct evidence that hypomethylating agents, such as 5-azaC, will lead to cancer. Although chronic injection of 5-azaC led to increased cancer in male Fischer rats (Carr *et al.*, 1984), injection of the deoxy analog 5-azadC, which is a more specific inhibitor of DNA methylation, results in reduction of tumorigenicity well below control levels (Carr *et al.*, 1988). These latter results have been overlooked because of the prevailing hypothesis that hypomethylation is involved in the carcinogenic process. Recently, 5-azadC treatment has been shown to prevent the initiation of neoplasia in a mouse model of APC, supporting the hypothesis that hypermethylation plays a role in neoplasia and contradicting the hypothesis that pharmacological hypomethylation can lead to neoplasia (Laird *et al.*, 1995). In contrast to the experiments by Laird and Jaenisch (1994), who showed that initiation of neoplasia could be inhibited by 5-azadC, MacLeod and Szyf (1995) previously had shown that the transformed state of the Y1 adrenocortical cell could

be reversed by 5-azadC at concentrations that are not cytotoxic, suggesting that hypomethylation could reverse the transformed state. The experiments by Laird *et al.* (1995) also show that chronic and systemic exposure to a hypomethylation drug is not toxic *per se*. These results have very important therapeutic implications for future use of methylation inhibitors as anticancer agents.

It is interesting to note that, in parallel to this activity in the field of DNA methylation, 5-azaC and 5-azadC were used previously in cancer therapy with some success as traditional nucleoside analogues. Clinical studies involving 5-azaC began in Europe in 1967 and in the United States in 1970 (for a review, see Von Hoff *et al.*, 1976). In a Phase II clinical study, 5-azaC had a transient antitumor effect on 17% of evaluable patients with carcinoma of the breast, 21% of patients with malignant lymphomas, and occasional responses were seen with a variety of solid tumors (Weiss *et al.*, 1977). However, the responses were transient, and it was believed that it was of minimal value as a single antitumor agent in solid tumors. However, 5-azaC showed consistent antitumor activity in patients with acute myelogenous leukemia resistant to previous treatment (Von Hoff *et al.*, 1976). Following the demonstration of the "differentiating" potential of 5-azaC (Jones, 1985; Taylor, 1993), it was considered as a potential therapy for leukemias that were blocked in their differentiation program (Wilson *et al.*, 1983; Taylor, 1993). A recent supplement of the journal *Leukemia* (1993) has been dedicated to some of the preclinical and clinical results obtained with 5-azaC in leukemias, and the results were encouraging. However, 5-azaC exhibits classic side effects of nucleoside analog therapy, such as nausea, vomiting, leukopenia, and hepatic toxicity, ranging from abnormal findings in liver function tests to hepatic coma (reviewed by Von Hoff *et al.*, 1976; Pinto and Zagonel, 1993). Very severe toxic side effects of 5-azaC were reported in a Phase II clinical study carried out by the Southwest Clinical Oncology Group in 1977; 13 deaths were attributable to drug toxicity (Quagliana *et al.*, 1977). The toxic side effects of 5-azaC limit its potential value either as a therapeutic or research tool.

It is now clear also that there is very limited support for the hypothesis that pharmacological hypomethylation is carcinogenic. The working hypothesis in this review proposes that induction of both DNA MeTase and demethylase activities is part of an oncogenic program. This can explain the seemingly conflicting observations of both hypomethylation and hypermethylation in oncogenesis. Moreover, it provides us with a therapeutic paradigm. If induction of these enzymes is part of the oncogenic program, inhibiting these enzymes should result in inhibition or reversal of the transformation process. Unfortunately, specific antagonists of DNA MeTase that do not exhibit serious side effects are not available yet. The pharmacological implication of this understanding is that both cancer and a large number of previously identified and novel targets for demethylation therapy could benefit from the development of novel inhibitors of DNA MeTase. The design and the utilization of such inhibitors requires a better understanding of the mechanisms regulating DNA

MeTase activity in the normal state and hypermethylation in cancer.

6.4. Inhibitors of Methylation as Pharmacological Switches of Cellular Epigenetic Programs

The picture that emerges from our discussion is that DNA methylation patterns are an important, but not the only, element controlling genomic programs. If the level of DNA MeTase activity is one critical control of the pattern of methylation, partial inhibition of DNA MeTase activity should result in a switch in genomic programs. The nature of the change will be dictated by other components controlling the genomic program. Moreover, mechanisms regulating the expression of DNA MeTase most probably will ensure that the loss of DNA MeTase activity will be compensated, such as has been observed previously following 5-azaC treatment (Gasson *et al.*, 1983). Because activation of genomic functions will require the presence of an array of cellular factors, demethylation will activate only functions that are preprogrammed to be expressed by the availability of transcription factors. This can explain why partial inhibition of methylation in predifferentiated cells does not result in a chaotic change in phenotype but, rather, a programmed move to the next stage in differentiation (Jones, 1985; Szyf *et al.*, 1992). Moreover, because the pattern of methylation is determined, as suggested, by signals in the DNA and proteins that interact with these signals (Szyf, 1991), the pattern of methylation probably would be restored to the original state in most, or almost all, sequences. It is expected, therefore, that systemic demethylation will have minor effect on programmed somatic cells. However, it should be effective in reactivating latent programs, such as activating fetal globin genes or activating a parentally imprinted gene or an exogenously-introduced gene, as suggested in Sections 6.1 and 6.2 (Fig. 8). In all of these cases, the transcription machinery is present in the cell. Thus, we predict that methylation inhibitors will have limited effects on somatic tissues, but dramatic effects on embryos, and should not be used during pregnancy. Experimental support for this hypothesis comes from the results with DNA MeTase knockout mice (Li *et al.*, 1992), on one hand, and the fact that systemic chronic treatment of mice with 5-azadC had no detectable toxic effects, on the other hand (Laird *et al.*, 1995).

The concept that the level of DNA MeTase expression can control a genomic program is especially critical in cancer, where it has been shown that oncogenic signaling leads to activation of the DNA MeTase (MacLeod *et al.*, 1995), which is suggested to drive the cell towards an oncogenic program. Thus, inhibition of DNA MeTase activity should result in restoring the original program (MacLeod and Szyf, 1995). If the hypothesis presented in this review is correct, partial inhibition of DNA MeTase activity should be a rational correction of the aberrant genomic program of cancer cells.

6.5. Inhibitors of DNA Methyltransferase

Inhibitors of DNA MeTase should have wide-ranging thera-

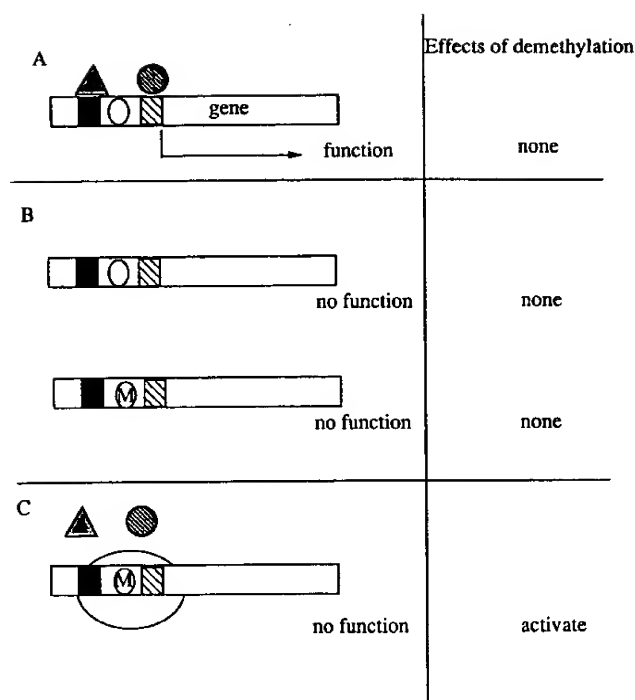


FIGURE 8. Effects of inhibition of DNA MeTase on gene expression. We predict, based on previous observations, that inhibition of DNA MeTase activity will result only in a limited and programmed change in gene expression. Gene expression is regulated by *cis*-acting sequences (promoter elements indicated by the hatched boxes and enhancers indicated by the shaded boxes) located in proximity to the coding sequences, transcription factors that interact with them (indicated by the shaded triangles and circles), and the state of methylation. An open oval indicates binding of a repressor complex (that might be composed of MDBPs). A methylatable site is indicated by an open circle, a methyl group is indicated as M. Demethylation will activate only a certain subset of genes that are programmed to be active by the presence of transcription factors in the cell, but are denied access to the transcription factors by DNA methylation.

peutic applications, as discussed in Section 6.4. The only specific inhibitor of DNA MeTase that is available currently is 5-azadC, which is phosphorylated by cellular kinases, incorporated into DNA, and traps DNA MeTase molecules by forming a covalent bond with the catalytic site of the protein (Fig. 9). This mechanism of action, although very efficient in inhibiting DNA methylation, can result in poten-

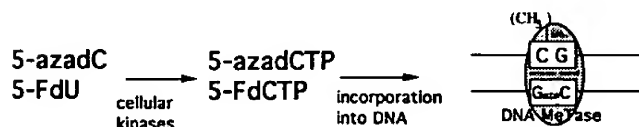


FIGURE 9. Inhibitors of DNA MeTase. Standard inhibitors of DNA MeTase: 5-azadC and 5-fluorodeoxyuridine (5-FdU). Both inhibitors are modified by cellular enzymes into 5-FdCTP or 5-azadCTP, and are incorporated into DNA during DNA replication. After they are incorporated in DNA, they form a covalent bond with the DNA MeTase; thus, trapping the protein and limiting the number of free DNA MeTase molecules available for methylating DNA.

tial side effects that limit the utility of 5-azadC as a therapeutic agent, as well as a research tool. First, 5-azadC is incorporated into the genome; the effects of the presence of nucleoside analogues such as 5-azadC on genomic functions are still unknown. For example, it might have an impact on genomic stability and have mutagenic effects (Zimmermann and Scheel, 1984; Katz, 1985; Call *et al.*, 1986; Amacher and Turner, 1987; Lal *et al.*, 1988; Osgood and Seward, 1989; Djalali *et al.*, 1990). Incorporation of 5-azadC into the genome might inhibit the ability of different DNA-binding proteins to interact with their cognate recognition sequences. Second, Juttermann *et al.* (1994) recently have shown that incorporation of 5-azadC into DNA causes trapping of bulky DNA MeTase molecules onto DNA, which might be responsible for the toxicity of the drug. Third, 5-azadC is a nucleoside analogue that might have a potential inhibitory effect on enzymes utilizing cytidine and its phosphorylated metabolites or precursors, such as orotidine monophosphate pyrophosphorylase (Guha, 1984), cytidine kinase (Liacouras and Anderson, 1979) and DNA polymerase. Fourth, 5-azadC could be converted to the ribo-azaC form, and inhibit RNA methylation in addition to DNA methylation (Lu *et al.*, 1976). Inhibitors of DNA MeTase that do not require additional metabolism in the cell and are not incorporated into DNA should be developed. Different strategies could be utilized to develop such inhibitors. First, antisense oligonucleotides to the DNA MeTase mRNA could be used to limit the availability of DNA MeTase in the cell (Szyf *et al.*, 1992; MacLeod and Szyf, 1995). Another approach is the use of analogues of the substrates of the DNA MeTase: SAM and CpG-containing DNA. The availability of the crystal structure of the bacterial HhaI CpG MeTase (Cheng *et al.*, 1993), and the striking homology between mammalian and bacterial CpG methylases (Kumar *et al.*, 1994), will enable the rational design of such inhibitors. Analogues of both substrates have been shown to inhibit DNA MeTase and DNA methylation. S-Adenosylhomocysteine (SAH), an analogue of SAM and one of the products of the methylation reaction, is an inhibitor of DNA methylation (Mixon and Dev, 1983). However, SAH and its analogues will inhibit a large number of different methylation reactions in the cell and must have non-specific side effects (Vedel *et al.*, 1978; Papadopoulos *et al.*, 1987). An additional approach that has been shown to be effective in inhibiting DNA methylation is the use of inhibitors of SAH hydrolysis, such as periodate-oxidized adenosine (Liteplo and Kerbel, 1986) or 3-deazaadenosine analogues (Aarbakke *et al.*, 1986; Chiang *et al.*, 1992). However, non-specific side effects are expected because of the predicted effects on all cellular methylation reactions. A double-stranded oligonucleotide bearing 5-fluorocytidine replacing cytidine in the CG recognition sequence is a mechanism-driven inhibitor of the DNA MeTase (Osterman *et al.*, 1988). This oligo has not been tested *in vivo* and the presence of the fluoride molecule might have some deleterious effects.

General inhibition of DNA MeTase is the rational approach in those cases where overexpression of DNA MeTase is involved in the mechanism of the disease. Other

cases, such as thalassemia and activation of exogenous genes in gene therapy, will require specific hypomethylation of discrete sites. Arguments were presented above to suggest that a general inhibition of DNA MeTase can result in specific changes in methylation patterns. An example is the specific hypomethylation of γ -globin in sickle cell anaemia patients treated with 5-azaC (Ley *et al.*, 1982). However, it is of obvious advantage to be able to hypomethylate and activate specific sequences. To address this goal, future experiments will be required to identify the *cis*-signals and *trans*-acting factors regulating specific methylation patterns, as discussed in Sections 5.4 and 5.6. Inhibitors of these *trans*-acting factors and analogues of their recognition sequences could then be designed. In the near future, the identification of *cis*-acting signals determining DNA methylation should enable the design of gene therapy vectors that could avoid the inactivating effects of *de novo* methylation.

7. SUMMARY

DNA methylation provides the genome with an additional level of information and enhanced memory of genetic programs. DNA methylation modulates genomic functions by affecting DNA-protein interactions. The DNA methylation pattern is controlled by an interplay of *cis*-acting signals, *trans*-acting factors, and the level of DNA MeTase and demethylation activities. The level of DNA MeTase and demethylase activities are controlled by cellular signaling pathways that are also involved in cancer. A general increase in DNA MeTase is mediated by the oncogenic Ras signaling pathway, and recent results suggest that it might play a causal role in carcinogenesis. This understanding of the regulation of DNA MeTase points to a new candidate for anticancer therapy. Novel inhibitors of DNA MeTase should be developed to realize this potential.

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